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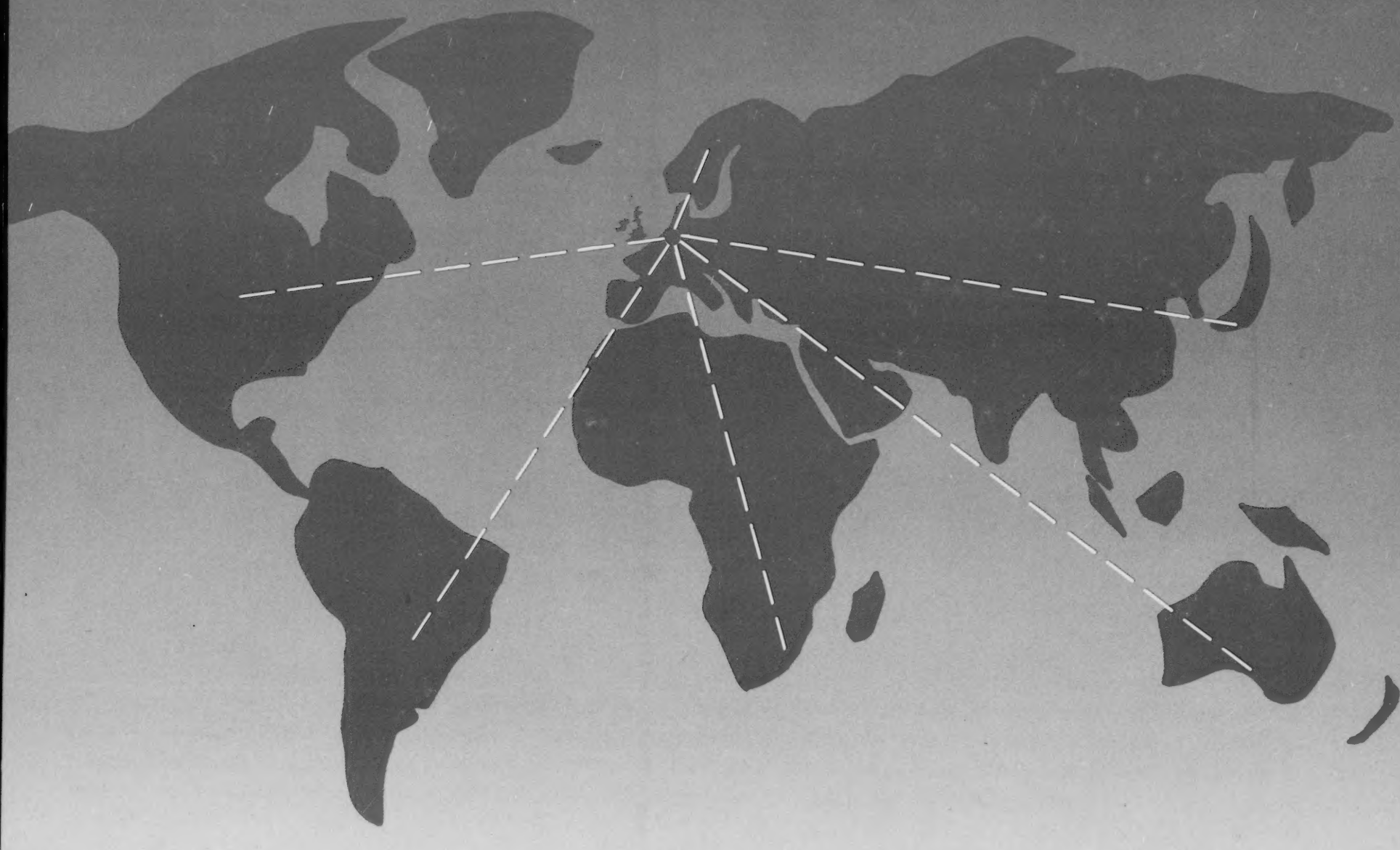

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The effects of muzolimine and urine from muzolimine-treated rats on $Na^+K^+Cl^-$ cotransport in Madin–Darby canine kidney cells

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Muzolimine is a loop diuretic with an original chemical structure devoid of the acidic or sulfonamide group known to be necessary for an interaction with $Na^+K^+Cl^-$ cotransport. We studied the effects of urine from muzolimine-treated rats on the $Na^+K^+Cl^-$ cotransport-dependent ^{86}Rb influx in MDCK cells. $Na^+K^+Cl^-$ cotransport was inhibited by urine obtained 15 min (42% inhibition) and 60 min (49% inhibition) after muzolimine injection (50 μmol/kg i.v.). Muzolimine itself was not detectable in the urine. Probenecid (100 μmol/kg i.v.) suppressed both the diuretic effect of muzolimine and the inhibition of $Na^+K^+Cl^-$ cotransport by urine from muzolimine-treated rats. These results suggest that the diuretic effect of muzolimine is due to the metabolism of muzolimine into an active compound which inhibits $Na^+K^+Cl^-$ cotransport after its secretion into the tubular lumen via a proximal pathway. The direct effect of muzolimine on $Na^+K^+Cl^-$ cotransport in MDCK cells was also tested: surprisingly, the inhibition of ^{86}Rb influx was significant in the presence of muzolimine (IC_{50} = 1.44 μM). We show that this effect was due to the metabolism of muzolimine by these cells into an active compound.

$Na^+K^+Cl^-$ cotransport; MDCK cells; Muzolimine; Loop diuretics

1. Introduction

Muzolimine is a loop diuretic which is chemically different from other diuretic drugs. In vivo, it has both the high-ceiling effect of loop diuretics and the long-lasting effect of thiazidic compounds (Meng et al., 1977). The site of action of muzolimine in the renal tubule is the thick ascending limb of the loop of Henle, as shown by free-water clearance measurements in rat and dog (Loew and Meng, 1977), stop-flow techniques in rats (Faedda et al., 1985) and microperfusion studies of the loop of Henle in rats (Greven and Köeling, 1983). But, in contrast to furosemide, the reference for loop diuretics, muzolimine does not affect NaCl absorption from the luminal side of the loop of Henle but appears to be effective from the peritubular side only (Faedda et al., 1985). It has also been shown that muzolimine modifies ion transport in the late distal tubule of the Necturus (Planelles et al., 1988). The precise way by which muzolimine acts at the cellular level is still subject to discussion. Muzolimine does not contain a sulfonamide or an acidic group, which appears to be necessary for an interaction with $Na^+K^+Cl^-$ cotransport (Greger and Wangemann, 1987), and muzolimine only inhibits $Na^+K^+Cl^-$ cotransport in red blood cells at high concentrations (Knorr et al., 1985).

Recently, it has been proposed that muzolimine may act via an active metabolite present in the urine (Greger and Wangemann, 1987; Imbs et al., 1987). This hypothesis was confirmed by the in vitro study of Wangemann et al. (1987), who showed that when urine from muzolimine-treated rats was perfused within the lumen of the rabbit thick ascending limb of the loop of Henle, the short-circuit current reflecting ion transport decreased. The authors suggested that muzolimine may be metabolised into an active compound present in the tubular lumen, which inhibits Na^+Cl^- transport in the loop of Henle.

In this study we investigated the action of muzolimine by measuring the effects of urine from piretanide- or muzolimine-treated rats on the loop diuretic-sensitive $Na^+K^+Cl^-$ cotransport reported in an established renal cell line: Madin-Darby canine kidney or MDCK (Rindler et al., 1984; Mc Roberts et al., 1982). The direct effect of muzolimine on $Na^+K^+Cl^-$ cotransport was also tested in these cells.

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2. Materials and methods

2.1. Materials

Piretanide, obtained from Hoechst-France (92800 Puteaux), was dissolved in dimethyl sulphoxide (DMSO). Muzolimine, obtained from Bayer AG (FRG, 5600 Wuppertal), was dissolved in a mixture of polyethyleneglycol and ethanol (2/3). Probenecid (Sigma-France, 38297 La Verpillière) was dissolved in 0.1 M NaOH. ^{86}Rb was obtained from Du Pont-New England Nuclear. Culture medium and foetal calf serum were purchased from Boehringer Mannheim-France (38240 Meylan). All other reagents were of analytical grade.

2.2. Rat treatment

Male Wistar rats (300 g, Janvier, St Berthevin, France) were anaesthetised with thiopenthal (50 mg/kg) and perfused with isotonic saline (NaCl 0.9%, 1 ml/kg per min) through a catheter in the right jugular vein; the bladder was also catheterised. The perfusion allowed urine osmolality (300 mOsm/l) and Na^+ content (140 mEq/l) to remain stable and isotonic with the plasma throughout the experiment. The solvents were administered (300 μl i.v. bolus) 120 min after perfusion began. After the effects of the solvents on diuresis had stabilised for 30-60 min, the various drugs were injected (300 μl i.v. bolus). Three rats were treated with piretanide (27 μmol/kg), a loop diuretic known to be secreted in the urine and to act by inhibiting $Na^+K^+Cl^-$ cotransport. Seven rats were treated with muzolimine (50 μmol/kg) and three rats received muzolimine (50 μmol/kg), followed 45 min later by probenecid (100 μmol/kg). Urine was collected for 15-min periods. The effects of several urine samples on $Na^+K^+Cl^-$ cotransport in MDCK cells were measured.

2.3. Cell culture

MDCK cells were a gift from Mrs F. Jaehnig (Laboratoire de Virologie, Faculté de Médecine, F-67085 Strasbourg) and come from a commercial MDCK cell line (Flow Laboratories, Les Ullis, France). MDCK cells were grown in Dulbecco-modified Eagle's medium, supplemented with glutamine (2 mM) and foetal calf serum (10%). Stock plates were kept in 75 cm^2 flasks. Confluent monolayers were subcultured by treatment with trypsin (0.05%) and EDTA (0.02%). The cells were plated at 1:3 dilution and incubated in a humidified 5% CO_2/95% O_2 air mixture at 37 °C. To measure $Na^+K^+Cl^-$ cotransport, cells were plated on 35 mm culture dishes at a density of 3.10^6 cells/cm^2 and were fed with fresh medium every three days. Under these conditions, cells were confluent on day 2; domes, which reflect functional ion transport, appeared on day 5; and cells were used for ^{86}Rb influx on day 7.

2.4. ^{86}Rb uptake

The ^{86}Rb influx in MDCK cells was measured using a modification of the technique described by Giesen-Crouse and McRoberts (1987). Culture dishes were washed three times at room temperature with 2 ml of assay buffer (mM: histidine 30, $MgSO_4$ 1, Na_2SO_4 15, KCl 30, sucrose 250, pH 7.2). Cells, on 35 mm culture dishes, were incubated for 90 min at room temperature with 1 ml of the same buffer containing 0.5 mM ouabain, with or without the urine samples or the desired concentration of piretanide or muzolimine. To measure the time course of muzolimine metabolism, muzolimine was added 5 or 30 min before the end of this preincubation period or at the beginning of the ^{86}Rb uptake period. At the end of the preincubation, the buffer was aspirated and replaced with 1 ml of the same buffer (+drug or urine), supplemented with ^{86}Rb (3 μCi/ml), and ^{86}Rb uptake was started at room temperature. Under these conditions, ^{86}Rb uptake was linear for 4 min. Uptake was terminated after 2 min by washing the cells three times with 3 ml of ice-cold buffer (mM: histidine 30, $MgSO_4$ 100, sucrose 227, pH 7.2). Then the plate was put on ice for 3 min to decrease extracellularly bound ^{86}Rb and washed again twice. Cells were dissolved in 1 ml sodium dodecyl sulfate (0.2%) and transferred to a counting vial containing 10 ml scintillation mixture (Packard TM 299). Radioactivity was measured with a Packard counter (0-1000 Kev). Each measurement of ^{86}Rb uptake was done in triplicate and every new cell culture series was tested for $Na^+K^+Cl^-$ cotransport with 1 and 100 μM piretanide. The difference between the ^{86}Rb influx in the absence of piretanide and the ^{86}Rb influx measured in the presence of 100 μM piretanide was considered to be the $Na^+K^+Cl^-$-dependent ^{86}Rb influx. Urine samples had to be diluted 100 times to avoid any increase in total ^{86}Rb influx caused by changes in the ion content of the incubation buffer.

2.5. Characterization of muzolimine in the urine

Urine samples obtained before or after muzolimine injection were treated with diethylene oxide (3 ml diethylene oxide/1 ml urine). Urine extracts were analysed for muzolimine by mass spectrometry (LKB 2091) and UV spectrometry (Beckman DU7 190-320 nm). The amount of muzolimine present in the urine after the injection of the diuretic was determined by comparison with control urine supplemented with concentrations of muzolimine from 1 μM to 1 mM and treated as described above.

2.6. Protein content

Protein content was determined with the method of Lowry (1951), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA, fraction V) as standard.

2.7. Statistics

The results are presented as means ± S.E.M. An analysis of variance for repeated measures was carried out (BMDP 2V, BMDP Statistical Software Inc, Cork, Ireland), followed by Scheffe's test. A P value < 0.05 was considered as significant.

3. Results

3.1. The effect of piretanide and muzolimine on $Na^+K^+Cl^-$ cotransport in MDCK cells

Under our experimental conditions, total ^{86}Rb influx in MDCK cells was 6.16 ± 1.12 nmol ^{86}Rb/min per mg protein (n = 10). The ^{86}Rb influx corresponding to $Na^+K^+Cl^-$ cotransport, identified in the presence of 100 μM piretanide, represented 92% of the total ^{86}Rb influx. The various solvents used for piretanide and muzolimine had no effect on ^{86}Rb influx. Figure 1 represents the dose-dependent inhibition of $Na^+K^+Cl^-$ cotransport in MDCK cells by piretanide and muzolimine. Surprisingly, muzolimine completely inhibited the $Na^+K^+Cl^-$-sensitive ^{86}Rb influx. The IC_{50}, which represents the dose inhibiting 50% of the ^{86}Rb influx corresponding to $Na^+K^+Cl^-$ cotransport, was 1.44 ± 1.21 μM (mean ± S.E.M., n = 5) for muzolimine, i.e. three times higher than for piretanide: 0.53 ± 0.32 μM (mean ± S.E.M., n = 5).

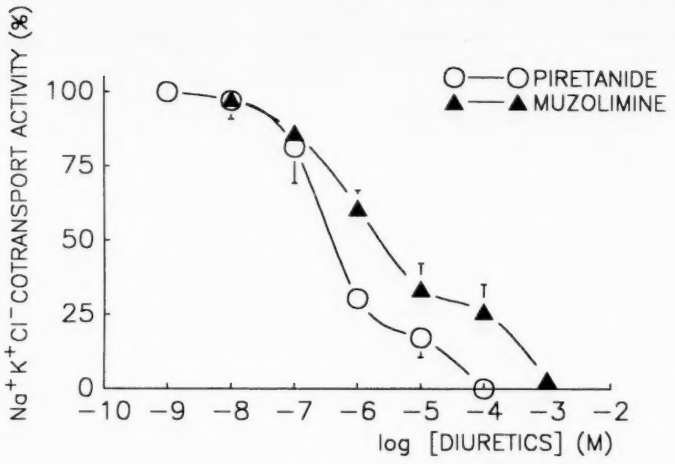

Fig. 1. Concentration-response curves for the inhibition of $Na^+K^+Cl^-$ cotransport in MDCK cells by piretanide (○) and muzolimine (▲). Each curve shows the means (±S.E.M.) of five determinations.

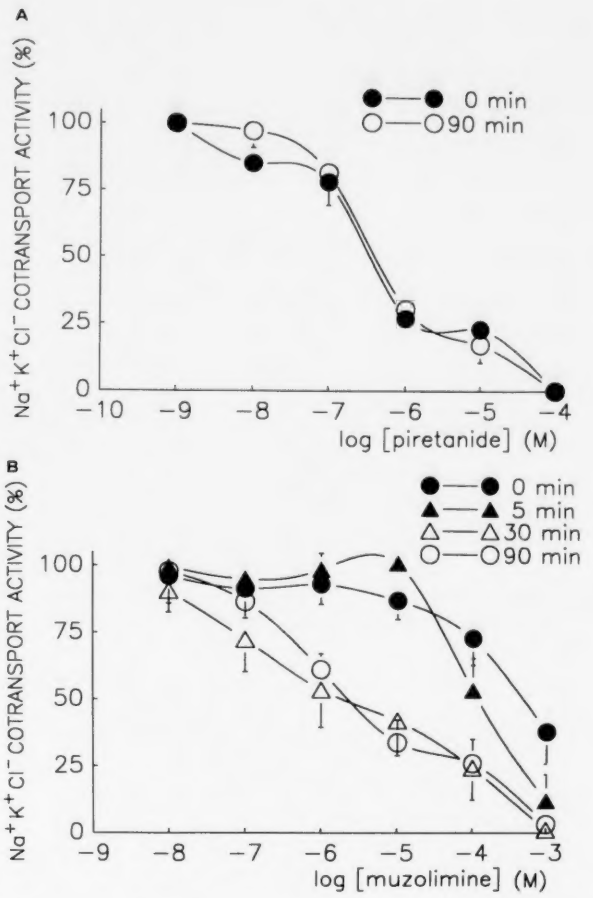

Fig. 2. Concentration-response curves for the inhibition of $Na^+K^+Cl^-$ cotransport in MDCK cells by piretanide (A) and muzolimine (B) after different preincubation times. Each curve shows the means (±S.E.M.) of three determinations.

3.2. Relationship between the length of the muzolimine preincubation and the inhibition of $Na^+K^+Cl^-$ cotransport

Until now, inhibition of $Na^+K^+Cl^-$ cotransport has only been observed with high concentrations (millimolar range) of muzolimine. Muzolimine lacks a sulfamide or acidic group, which appears to be necessary for an interaction with $Na^+K^+Cl^-$ cotransport (Greger and Wangemann, 1987).

This unexpected effect of muzolimine on $Na^+K^+Cl^-$ cotransport in MDCK cells could be due to the metabolism of muzolimine into an active compound during the 90-min preincubation period. To test this hypothesis, dose-effect curves for muzolimine or piretanide were determined at different preincubation times (fig. 2). When piretanide was added at the beginning of the uptake period, i.e. without preincubation, the dose-effect curve for piretanide was identical to the curve obtained after 90 min preincubation (fig. 2A). In contrast, without incubation, the IC_{50} for muzolimine (2B) was 0.77 + 0.62 mM (n = 3); after preincubation, the dose-effect curve for muzolimine was moved to the left. When muzolimine was added 5 min before the end of the preincubation period, the IC_{50} was 0.12 ± 0.07 mM (n = 3). After 30 min of preincubation with muzolimine, the dose-response curve was comparable

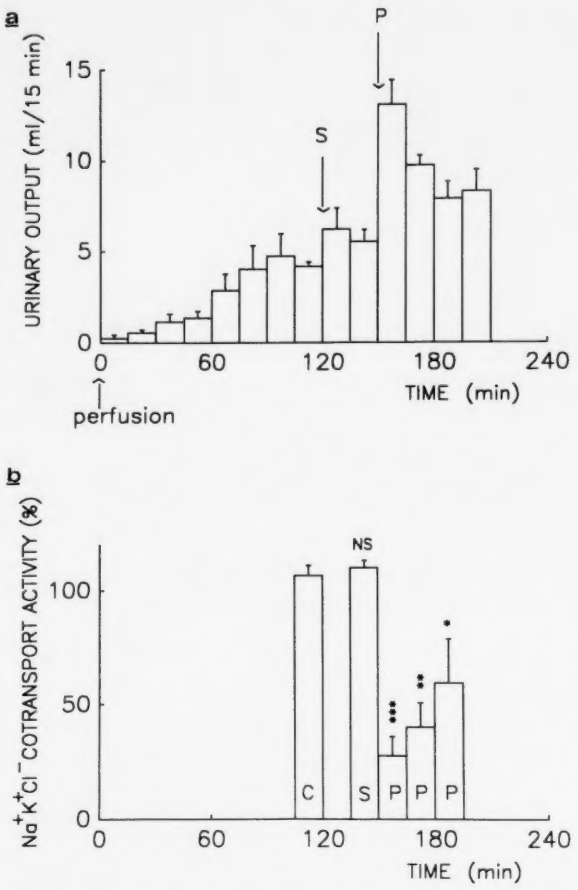

Fig. 3. (a) The effects of solvent (S) and piretanide (P, 27 μmol/kg) on rat urinary output as a function of perfusion time (NaCl 0.9%, 1 ml/kg per min). (mean $\pm$ S.E.M., n = 3). (b) The effects of corresponding urine samples (control (C), solvent (S), piretanide (P)) and $Na^+K^+Cl^-$ cotransport in MDCK cells (mean $\pm$ S.E.M., n = 3). ***,**,* Indicate statistical difference from control ($P < 0.001$, $P < 0.01$, $P < 0.05$, respectively). NS = non-significant.

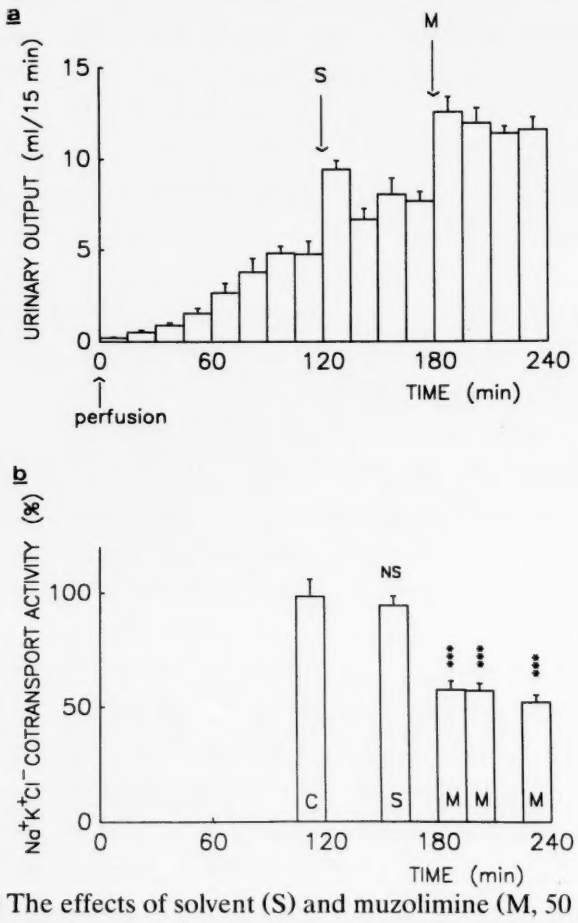

Fig. 4. (a) The effects of solvent (S) and muzolimine (M, 50 μmol/kg) on rat urinary output (mean $\pm$ S.E.M., n = 7). (b) The effects of corresponding urine samples (control (C), solvent (S), muzolimine (M)) on $Na^+K^+Cl^-$ cotransport in MDCK cells (mean $\pm$ S.E.M., n = 7). *** Indicates statistical difference from control ($P < 0.001$). NS = non-significant.

to that obtained after 90 min with an $IC_{50} = 1.27 \pm 1.3$ μM.

3.3. The effect of urine from piretanide-treated rats on $Na^+K^+Cl^-$ cotransport in MDCK cells

Figure 3 shows the effect of piretanide on urine volume (3a) and the corresponding effects of several urine samples on $Na^+K^+Cl^-$ cotransport in MDCK cells (3b). The saline perfusion elicited a large diuresis which increased briefly after the injection of the piretanide solvent. Piretanide itself produced a large and rapid increase in urine volume but this effect was short-lived. Urine from untreated or solvent-treated rats had no inhibitory effect of $Na^+K^+Cl^-$ cotransport. In contrast, after piretanide injection, all urine samples decreased $Na^+K^+Cl^-$ cotransport. The greatest decrease, down to 28%, in total $Na^+K^+Cl^-$ cotransport was observed with the first urine sample taken after piretanide injection. This effect was short-lived: 45 min after piretanide injection, $Na^+K^+Cl^-$ cotransport reached 59% of the total.

3.4. The effect of urine from muzolimine-treated rats on $Na^+K^+Cl^-$ cotransport in MDCK cells

Figure 4 shows the effect of muzolimine on rat diuresis (4a) and the corresponding effects of several urine samples on $Na^+K^+Cl^-$ cotransport (4b). Compared to piretanide, muzolimine induced a moderate but long-lasting increase in urine volume. After muzolimine injection, all the urine samples decreased $Na^+K^+Cl^-$ cotransport, which was 58% of the total transport 15 min after muzolimine injection. This effect was stable during the hour following the injection of muzolimine.

3.5. The effect of urine from muzolimine- and probenecid-treated rats on $Na^+K^+Cl^-$ cotransport in MDCK cells

Figure 5 shows the effect of combined muzolimine and probenecid treatment on rat diuresis (5a) and on $Na^+K^+Cl^-$ cotransport (5b). Probenecid itself had no effect on diuresis or on $Na^+K^+Cl^-$ cotransport (data not shown). In this series of experiments, the effect of muzolimine on diuresis and the effects of urine from muzolimine-treated rats on $Na^+K^+Cl^-$ cotransport were analogous to those shown in fig. 3. After the injection of probenecid, both the diuretic effect of muzolimine and the effect of urine on $Na^+K^+Cl^-$ cotransport disappeared within 15 min.

3.6. The characterization of muzolimine in the urine

No muzolimine was detected in the urine after muzolimine injection, by either UV spectrometry or mass

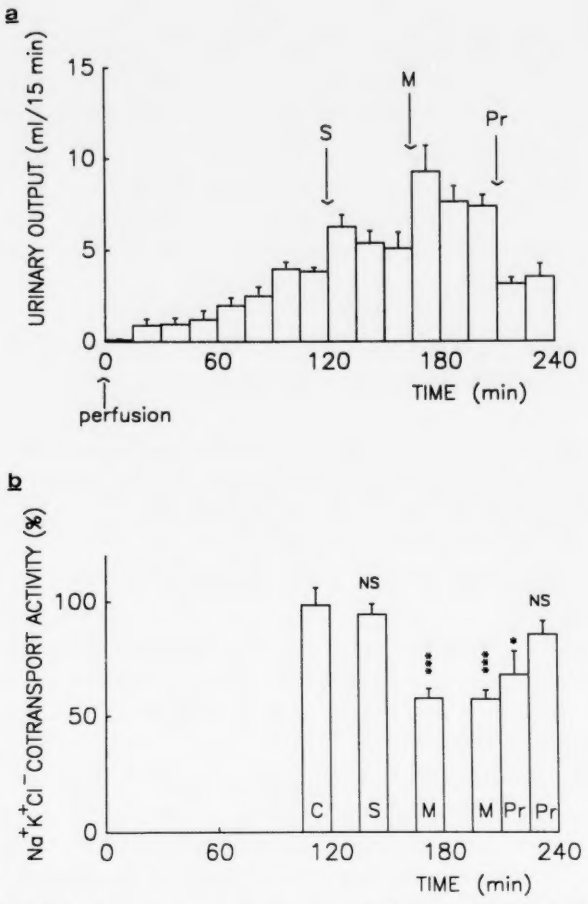

Fig. 5. (a) The effects of muzolimine (M) and probenecid (Pr) on rat urinary output (mean ± S.E.M., n = 3). (b) The effects of corresponding urine samples (control (C), solvent (S), muzolimine (M), probenecid (Pr)) on $Na^+K^+Cl^-$ cotransport in MDCK cells (mean ± S.E.M., n = 3). ***·**·* Indicate statistical difference from control ($P < 0.001$, $P < 0.01$, $P < 0.05$, respectively). NS = non-significant.

spectrometry. The limit of detection of both techniques was 10 μM.

4. Discussion

MDCK cells have a $Na^+K^+Cl^-$ cotransport which is sensitive to loop diuretics (Rindler et al., 1982; McRoberts et al., 1982). ^{86}Rb influx is a validated way of evaluating this cotransport. Under similar experimental conditions, the total ^{86}Rb influx measured in this study was comparable to that obtained by Giesen-Crouse and McRoberts (1987) but the $Na^+K^+Cl^-$-sensitive ^{86}Rb uptake in our MDCK cells represented more than 90% of total uptake, which was more than the 50% obtained by Giesen-Crouse and McRoberts in their cells. This result allows us to conclude that the observed inhibitory effect of muzolimine is the result of an inhibition of $Na^+K^+Cl^-$ cotransport. The IC_{50} measured in our study for piretanide was also in line with the results of other studies, including those of ^{86}Rb influx or binding in MDCK cells (Giesen-Crouse and McRoberts, 1987), or on kidney membranes (Giesen-Crouse et al., 1985).

The observed inhibitory effect of muzolimine on $Na^+K^+Cl^-$ cotransport in MDCK cells was surprising and raises a number of questions. Several approaches have demonstrated that muzolimine acts differently from loop diuretics such as furosemide or piretanide: muzolimine (up to 10^{-4} M) does not inhibit salt transport in an isolated rabbit ascending limb of the loop of Henle (Schlatter et al., 1983), it does not displace the binding of [^{3}H]piretanide on dog kidney membrane ($IC_{50} > 1$ mM, Imbs et al., 1986) and it only inhibits $Na^+K^+Cl^-$ cotransport in human red blood cells with a high IC_{50} (1.98 mM) (Knorr et al., 1985). Moreover, studies of the correlation between the chemical structure of loop diuretics and their inhibitory potencies on salt transport in the loop of Henle (Schlatter et al., 1983; Wittner et al., 1987) suggest that the presence of an acidic group and a sulfonamide group is required for an interaction with $Na^+K^+Cl^-$ cotransport. Muzolimine does not contain any of these groups. The inhibitory effects observed in our study could have been due to the transformation of muzolimine during storage, but mass spectrometry showed no impurities in the muzolimine. Moreover, in a preliminary study, we confirmed that the same sample of muzolimine that inhibited $Na^+K^+Cl^-$ cotransport in MDCK cells did not inhibit the binding of [^{3}H]piretanide on dog kidney membranes, which have been described as a model to screen for compounds affecting $Na^+K^+Cl^-$ cotransport (Giesen-Crouse et al., 1988). We suggest that the observed direct inhibitory effect of muzolimine on MDCK cells is due to the ability of these cells to transform muzolimine into an active compound during the incubation which preceded the measurement of ^{86}Rb influx. This hypothesis was confirmed by the results obtained when studying the relationship between the length of the muzolimine incubation period and the observed inhibitory effect on $Na^+K^+Cl^-$ cotransport. Whereas in the case of piretanide no preincubation was necessary to induce a maximal effect, it was clear that without preincubation muzolimine had only a weak effect on $Na^+K^+Cl^-$ cotransport, with an IC_{50} comparable to that obtained in other experimental models such as red blood cells (Knorr et al., 1985) or binding of [^{3}H]piretanide on dog kidney membranes (Imbs et al., 1986). After a preincubation, the dose-response curves were moved to the left with decreasing IC_{50} values, which suggests that there was a more active compound present. No differences were recorded between a 30- and a 90-min preincubation period. This suggests that the full capacity of MDCK cells to transform muzolimine was obtained within 30 min.

In the second part of this study, we examined the effects of urine from piretanide- or muzolimine-treated rats on $Na^+K^+Cl^-$ cotransport in MDCK cells. Piretanide is excreted in the urine by an active anion transporter and it is therefore concentrated in the lumen. This transport system can be inhibited by probenecid. The urine (diluted 100 times) from pire-

tanide-treated rats inhibited 70% of $Na^+K^+Cl^-$ cotransport in MDCK cells. The effect of $Na^+K^+Cl^-$ cotransport parallels the diuretic effect of piretanide. Urine from muzolimine-treated rats also inhibited $Na^+K^+Cl^-$ cotransport. Muzolimine itself was probably not responsible for the effect of the urine since muzolimine was not detected in the urine. The limit of detection of the methods used (UV spectrometry, mass spectrometry) was 10 μM, but taking into account the 100-fold dilution of the urine, no more than 0.1 μM of muzolimine would have been in contact with MDCK cells. According to fig. 1, a dose of 0.1 μM induced only 10-15% inhibition while a 50% inhibition was observed with urine obtained after muzolimine administration. Our results agree with those of Wangemann et al. (1987) who observed a 50% inhibition of the short-current circuit in an isolated ascending limb of the loop of Henle with 150-fold-diluted urine from muzolimine-treated rats (70 μmol/kg). On the other hand, probenecid completely inhibited the diuretic effect of muzolimine. This result differs from that of Wangemann et al. (1987), who showed the persistence of a small but significant diuretic effect of muzolimine after probenecid treatment. In our study, probenecid also abolished the inhibitory effect on $Na^+K^+Cl^-$ cotransport observed with the corresponding urine; this suggests that the compound involved in both effects was one with an acidic group, secreted in the urine by the probenecid-sensitive anion transporter in the proximal tubule.

In conclusion, this study confirms that the diuretic effect of muzolimine is mainly due to an active metabolite and provides new data on the mechanism of action of this metabolite which, like furosemide or piretanide, inhibits Na^+K^+Cl cotransport in the ascending limb of the loop of Henle from the luminal side. The metabolism of muzolimine into an active compound may explain the long-lasting effects of this diuretic. We showed that MDCK cells are also able to transform muzolimine into an active compound. Further studies will be necessary to identify these two compounds and to establish whether there is one or several active metabolites of muzolimine.

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References

Faedda, R., A. Satta, F. Branca, B. Contu and E. Bartoli, 1985, Demonstration of action of muzolimine on the serosal side of Henle's loop cells, Z. Kardiol. 74 (Suppl. 2), 166.

Giesen-Crouse, E., C. Welsch, J.L. Imbs, M. Schmidt and J. Schwartz, 1985, Characterization of a high affinity piretanide receptor on kidney membranes, Eur. J. Pharmacol. 144, 23.

Giesen-Crouse, E. and J.A. McRoberts, 1987, Coordinate expression of piretanide receptors and $Na^+K^+Cl^-$ cotransport activity in Madin-Darby Canine Kidney cell mutants, J. Biol. Chem. 262, 17393.

Giesen-Crouse, E., P. Fandeleur, C. Welsch, H. Englert, H.J. Lang, M. Schmidt and J.L. Imbs, 1988, Binding of loop diuretics to their renal receptors: use a screening model for potential diuretic activity, Fundam. Clin. Pharmacol. 2, 145.

Greger, R. and P. Wangemann, 1987, Loop diuretics, Renal Physiol. (Basel) 10, 174.

Greven, J. and B. Kölling, 1983, Effects of muzolimine in microperfusion studies of loops of Henle in rats, in: Recent Advances in Diuretic Therapy, ed. V.E. Andreucci (Experta Medica, Amsterdam) p. 37.

Imbs, J.L., M. Schmidt and E. Giesen-Crouse, 1987, Pharmacology of loop diuretics, Adv. Nephrol. 16, 137.

Knorr, A., B. Garthoff, A. Ingendoh and M. De Mendonça, 1985, Influence of muzolimine and other diuretics on human red cell Na^+,K^+-cotransport, Z. Kardiol. 74 (Suppl. 2), 175.

Loew, D. and K. Meng, 1977, The renal mechanism of muzolimine, Pharmatherapeutica 1, 333.

Lowry, O.H., N.H. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

McRoberts, J.A., S. Erlinger, M.J. Rindler and M.H. Saier, Jr., 1982, Furosemide-sensitive salt transport in the Madin-Darby Canine Kidney cell line, J. Biol. Chem. 257, 2260.

Meng, K., D. Loew, K. Stoepel and F. Hoffmeister, 1977, Pharmacology of muzolimine, a long-acting, high-ceiling diuretic, Curr. Med. Res. Opioid 4, 556.

Planelles, G., B. Meilhac and T. Anagnostopoulos, 1988, Effects of muzolimine on the late distal tubule of necturus kidney, J. Pharmacol. Exp. Ther. 246, 1152.

Rindler, M.J., J.A. McRoberts and M.H. Saier, Jr, 1982, (Na^+, K^+)-cotransport in the Madin-Darby Canine Kidney cell line, J. Biol. Chem. 257, 2254.

Schlatter, E., R. Greger and C. Weidtke, 1983, Effect of 'high-ceiling' diuretics on active salt transport in the cortical thick ascending limb of Henle's loop of rabbit kidney, Pflügers, Arch. 396, 210.

Wangemann, Ph., R. Braitsch and R. Greger, 1987, The diuretic effect of muzolimine, Eur. J. Physiol. 41, 674.

Wittner, M., A. DiStefano, P. Wangemann, J. Delarge and R. Greger, 1987, Analogues of torasemide-structure function relationship-experiments in the thick ascending limbs of the loop of Henle of rabbit nephron, Pflügers Arch. 408, 54.

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A comparison of endothelium-derived relaxing factor activity in the coronary and renal arteries of the pig

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A cascade bioassay system has been used to quantify the basal and receptor-mediated endothelium-derived ralaxing factor (EDRF) activity of pig coronary artery and pig renal artery. When exposed to EDRF released from a common donor vessel, pig coronary artery smooth muscle showed a greater sensitivity to EDRF than pig renal artery, and these differences were paralleled by differential responses to sodium nitroprusside. When mounted as donors in the bioassay, and EDRF detected using a common recipient, pig coronary artery and renal artery endothelium showed similar basal EDRF release rates. EDRF release in response to substance P was greater from pig coronary artery donors, but EDRF release in response to bradykinin was greater from pig renal artery donors. The data indicate that differences in EDRF response and EDRF release occur in different vessels, and that certain EDRF-releasing agents may exert regional vasodilator effects.

EDRF (endothelium-derived relaxing factor); Coronary artery; Renal artery; Bradykinin; Substance P

1. Introduction

Differences in basal endothelium-derived relaxing factor (EDRF) activity between vessels in the same animal have been reported by Griffith and colleagues (1984) who showed high basal EDRF activity in rabbit coronary but not aortic preparations, and Collins and coworkers (1986), who demonstrated that agonist-induced Ca^{2+} influx is inhibited more by basal EDRF activity in dog isolated coronary artery preparations than in rabbit aorta.

Receptor-mediated EDRF responses also vary according to artery type. In a controlled comparative study using isolated arterial ring preparations from the dog, Angus et al. (1986a) showed that endothelium-dependent relaxation to both noradrenaline and substance P varied in the order coronary > carotid > femoral ≫ renal = mesenteric. More recently Angus and coworkers (1988) have extended this investigation to include other EDRF-releasing agents and have shown that relaxant responses to acetylcholine and ATP vary in the same order, but that bradykinin is unexpectedly potent in the renal artery. Comparison with the potency order of glyceryl trinitrate in the same series of vessels suggests that the responsiveness of the underlying smooth muscle is the major determinant of the potency of all the EDRF-releasing agents except bradykinin. In isolated pig arterial preparations (Angus et al., 1986b) EDRF responses in noradrenaline-constricted vessels were found to vary in the order coronary = mesenteric > renal > femoral = carotid, again suggesting heterogeneity.

These differences in EDRF activity could be due to variation in the amount of EDRF released by different arteries or variation in the response of the smooth muscle to EDRF. We have previously shown that both the smooth muscle response to EDRF (Christie and Lewis, 1988) and the amount of EDRF released in response to EDRF-releasing agents (Christie et al., 1989) differs between arterial preparations from different species.

The objectives of the present study were firstly to quantify the basal EDRF release from pig coronary artery and pig renal artery preparations. Secondly to quantify the receptor-mediated EDRF release in response to substance P and bradykinin. And thirdly to compare the sensitivity of the smooth muscle of pig coronary artery and pig renal artery to EDRF and sodium nitroprusside.

A bioassay cascade system was used to measure both the amount of EDRF released from endothe-

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lium-intact donors as detected by a common recipient, and the response of endothelium-denuded pig coronary artery and pig renal artery preparations to EDRF released from a common donor vessel. The results indicate that although basal EDRF release rates from the two arteries are similar, there are differences in both the receptor-mediated EDRF release from endothelium, and in the smooth muscle response to EDRF and sodium nitroprusside.

2. Materials and methods

2.1. Vessel preparation

The collection and preparation of vessels and the design of the bioassay used have been described in detail elsewhere (Christie and Lewis, 1988). Briefly, pig circumflex coronary arteries and the extrarenal portion of the renal arteries were collected from a local abattoir in pregassed (95% O_2; 5% CO_2) Holman's solution of the following composition (mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, D-glucose 11, sucrose 10. The Holman's solution also contained indomethacin (10^{-5} M) as a cyclooxygenase inhibitor to prevent prostanoid synthesis. Vessels were either carefully trimmed to 3 cm lengths for use as endothelium-intact donor vessels, or cut into 2-3 mm wide rings and denuded of endothelium for use as recipients in the bioassay.

2.2. Experimental protocol

Responses to EDRF and nitroprusside: endothelium-denuded rings of pig coronary artery and renal artery were mounted in parallel as recipients in the bioassay, perfused with Holman's solution (2 ml/min) at 37°C via a stainless steel tube mounted in the donor chamber, and isometric tension responses recorded on a Devices chart recorder. Pig coronary artery and renal artery rings were equilibrated at a resting tension of 5 and 4 g respectively, the optimum resting tension for constriction in each ring type (data not shown) with frequent readjusting until stress relaxation no longer occurred. After approximately 60 min they were preconstricted with prostaglandin (PG) $F_{2\alpha}$ (10^{-5} M) and tested for the absence of endothelium by brief (1 min) exposure to the calcium ionophore A23187 (10^{-7} M). Any rings showing a relaxant response to A23187 were discarded. After re-equilibration, cumulative concentration responses to $PGF_{2\alpha}$ (10^{-7}–3×10^{-5} M) were obtained and used to calculate an EC_{95} (the concentration of $PGF_{2\alpha}$ producing 95% of maximum constriction) for each ring. Rings were then washed and re-equilibrated before preconstriction with the EC_{95} for $PGF_{2\alpha}$ infused post-donor (i.e. immediately upstream of the rings) using an LKB peristaltic pump. As pig coronary arteries possess β-adrenoreceptors acebutolol (10^{-5} M) was also infused post-donor to abolish any β-mediated changes in smooth muscle tone.

When constrictor responses had stabilised, the recipient rings were perfused via an endothelium-intact pig coronary artery donor vessel. EDRF was released from the pig coronary artery by a supramaximal concentration of A23187 (10^{-6} M) infused pre-donor. After a maximum relaxant response to EDRF had been obtained the donor vessel was removed from the circuit and replaced with stainless steel tubing, $PGF_{2\alpha}$-induced tone was allowed to return, and cumulative concentration-responses to sodium nitroprusside obtained in the continued presence of A23187.

2.3. Release of EDRF in response to substance P and bradykinin

Endothelium-denuded pig coronary artery rings were mounted as recipients in the bioassay, perfused at 2 ml/min with Holman's solution. Flow rates were not altered according to donor dimensions as previously reported (Christie et al., 1989) as preliminary results indicated that basal EDRF release rates from pig coronary artery and pig renal artery donors at low flow rates were determined by the frequency of flow pulses, rather than the absolute shear stress, which was very low in this system. Perfusing both donor types at the same flow rate normalised both the frequency of flow pulses (2.0 Hz) and the the donor-recipient transit time (3.5 s). Recipient pig coronary artery rings were preconstricted with $PGF_{2\alpha}$ (at a concentration representing the EC_{95} value in each preparation) infused post-donor using an LKB pump. Acebutolol (10^{-5} M) was also infused post-donor to prevent the stimulation of smooth muscle β-receptors. After vasoconstrictor responses had stabilised, the donor vessel was flushed with Holman's solution at 2 ml/min for 3 min before being allowed to perfuse the recipient rings. Basal EDRF release, and EDRF release in response to 60 s pulses of substance P (10^{-11}-10^{-8} M) or bradykinin (10^{-9}-10^{-6} M) was measured. At the end of each infusion period of substance P or bradykinin a supramaximal concentration (10^{-6} M) of A23187 was added pre-donor to ensure that smooth muscle relaxation to EDRF released by substance P and bradykinin was sub-maximal. At the concentrations used, substance P, bradykinin and A23187 had no direct effect on the tone of the pig coronary artery recipient rings.

2.4. Calculations

Relaxation responses to EDRF and sodium nitroprusside were expressed as a percentage of the initial $PGF_{2\alpha}$-induced preconstriction, and EDRF responses

were quantified by reference to a sodium nitroprusside concentration-response curve. EDRF concentrations at the recipients, and EDRF release rates from the donor endothelium were calculated as previously described (Christie et al., 1989), using a value for the EDRF decay constant of -0.142 per s (corresponding to a decay half-life of 4.9 s in this system). EDRF release rates were then expressed as nU/cm^2 per s, where 1 nU/cm^2 per s = 1 cm^2 of endothelial cells producing the relaxing equivalent of 10^{-9} M of sodium nitroprusside in 1 s. Slopes and EC_{50} values were calculated from a line fitted to the straight part of the concentration-response curve by a least squares minimisation procedure. Results were analysed by Student's t-test and considered significant when $P < 0.05$. Data are presented as means $\pm$ S.E.M.

2.5 Drugs

Bradykinin (triacetate salt), calcium ionophore A23187 (mixed Ca^{2+} : Mg^{2+} salt), indomethacin, sodium nitroprusside and substance P (acetate salt) were purchased from the Sigma Chemical Co. Ltd., Poole, UK. Acebutolol hydrochloride was a gift from May & Baker Ltd., Dagenham, UK. Prostaglandin $F_{2\alpha}$ (Prostin) was purchased from Upjohn Ltd., Crawley, UK.

3. Results

3.1. Responses to EDRF and nitroprusside

Figure 1 shows typical tension recordings from endothelium-denuded pig coronary artery and renal artery rings. Note that the EDRF-induced relaxation of the pig coronary artery ring was greater than that of the renal artery ring. If a renal artery donor was used instead of a coronary artery donor, similar results were obtained (data not shown).

Figure 2 shows mean relaxant responses of pig coronary artery and renal artery preconstricted with $PGF_{2\alpha}$ (EC_{95}) to EDRF released from a pig coronary artery donor by A23187 (10^{-6} M). $PGF_{2\alpha}$ increased tension in the coronary artery and renal artery rings by 11.4 ± 0.6 g and $14.4 + 1.0$ g, which relaxed by 59 ± 8 and $25 \pm 8\%$, respectively, when exposed to EDRF. The response of the coronary artery rings to EDRF was significantly greater than the renal artery rings ($P < 0.01$).

Figure 3 shows concentration-response curves to relaxation induced by sodium nitroprusside in the same coronary artery and renal artery rings preconstricted with $PGF_{2\alpha}$ (EC_{95}) and in the continued presence of A23187 (10^{-6} M). The curve for the coronary artery rings has a slope of 34.2 ± 2.4 an EC_{50} value $2.5 \pm 0.2 \times 10^{-7}$ M, and an asymptote of $103 \pm 6\%$. The curve for the renal artery rings has a slope of 27.6 ± 2.7, an EC_{50} value $1.42 \pm 0.54 \times 10^{-6}$ M, and an asymptote of $88 \pm 5\%$. The EC_{50} value of the coronary artery was significantly less than that for the renal artery ($P < 0.01$). The maximum percentage relaxations of coronary artery and renal artery rings in response to EDRF (see fig. 2) are equivalent to the relaxations produced by $5.0 \pm 1.2 \times 10^{-7}$ and $2.5 \pm 0.6 \times 10^{-7}$ M sodium nitroprusside, respectively. These values are however not significantly different.

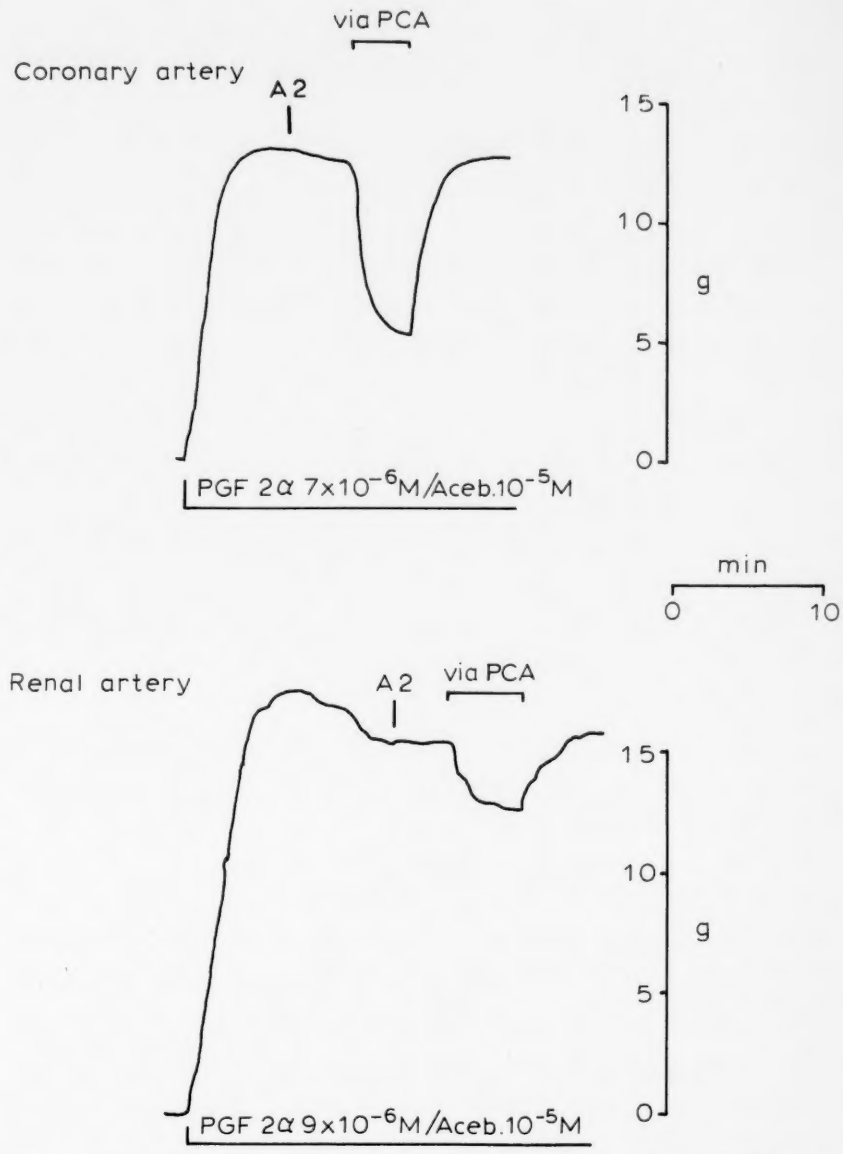

Fig. 1. Representative traces showing relaxation of a preconstricted endothelium-denuded pig coronary artery (PCA) (upper trace) and pig renal artery (PRA) (lower trace) induced by EDRF released with A23187 from a common endothelium-intact pig coronary artery, in a bioassay cascade. Note that the response to EDRF is greater in the pig coronary artery than the pig renal artery.

3.2. Release of EDRF in response to substance P and bradykinin

3.2.1. Substance P

Figure 4 shows typical tension recordings from β-blocked, endothelium-denuded pig coronary artery rings exposed to EDRF released from a pig coronary artery and pig renal artery donor. Note that substance

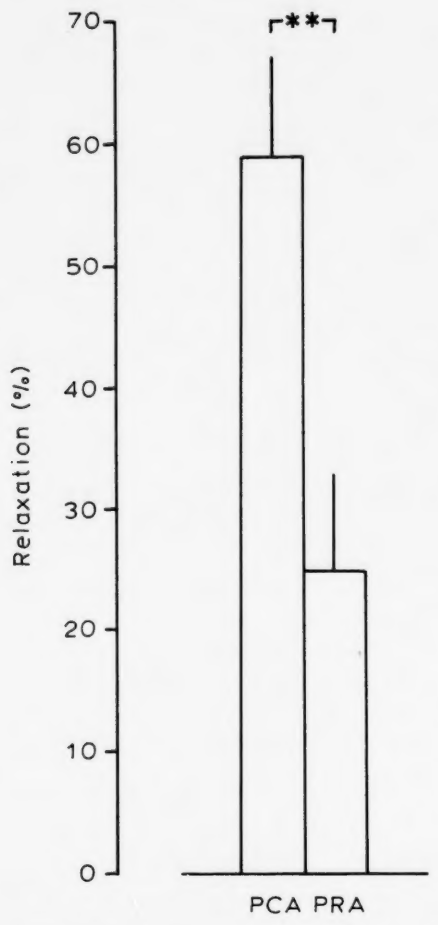

Fig. 2. Bar diagrams showing mean ± S.E.M. % relaxation of preconstricted pig coronary artery (PCA) and pig renal artery (PRA) to EDRF released from an endothelium-intact pig coronary artery by A23187, in a bioassay cascade. Relaxation in the PCA preparations was significantly greater than in the pig renal artery preparations (** $P < 0.01$; $n = > 10$).

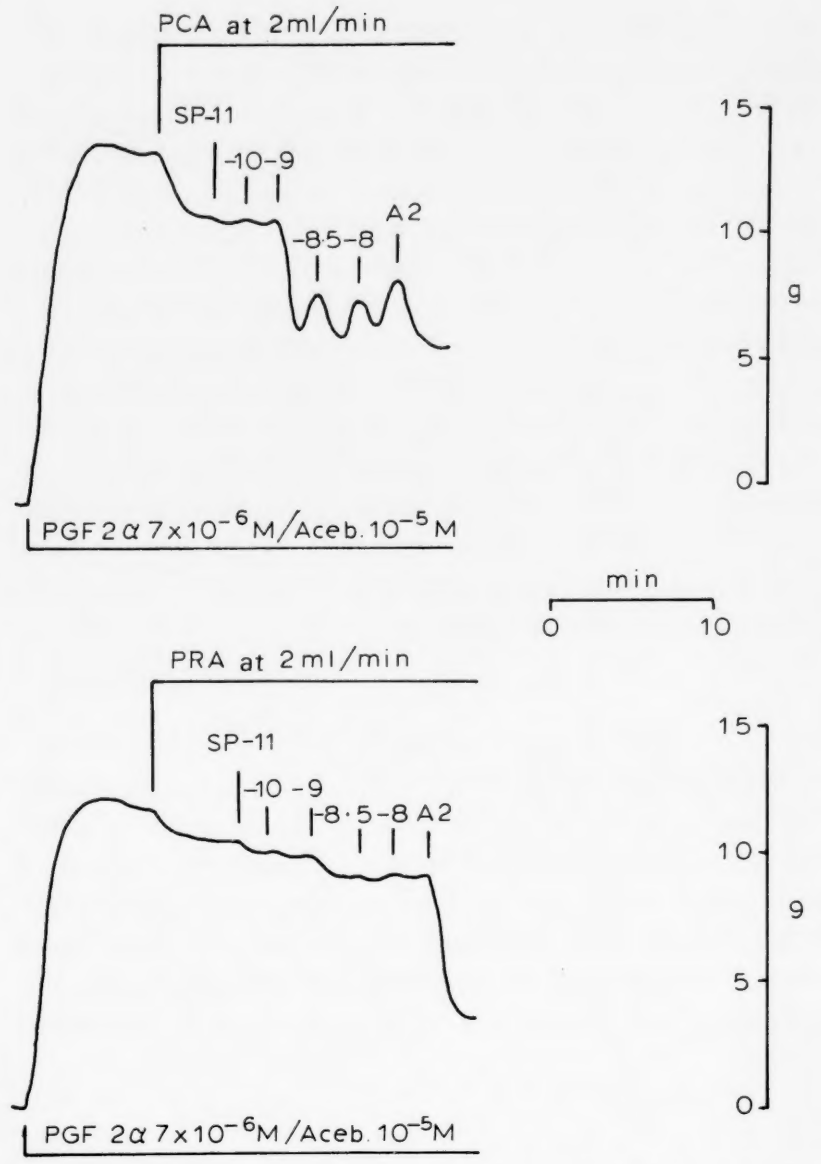

Fig. 4. Representative traces showing relaxation responses of preconstricted endothelium-denuded pig coronary artery to EDRF released by increasing concentrations of substance P (SP) and a supramaximal concentration of the calcium ionophore A23187 (A2), from an endothelium-intact pig coronary artery (PCA) (upper trace) or pig renal artery (PRA) (lower trace) in a bioassay cascade. Note that the relaxation responses were greater to EDRF released from the pig coronary artery than pig renal artery.

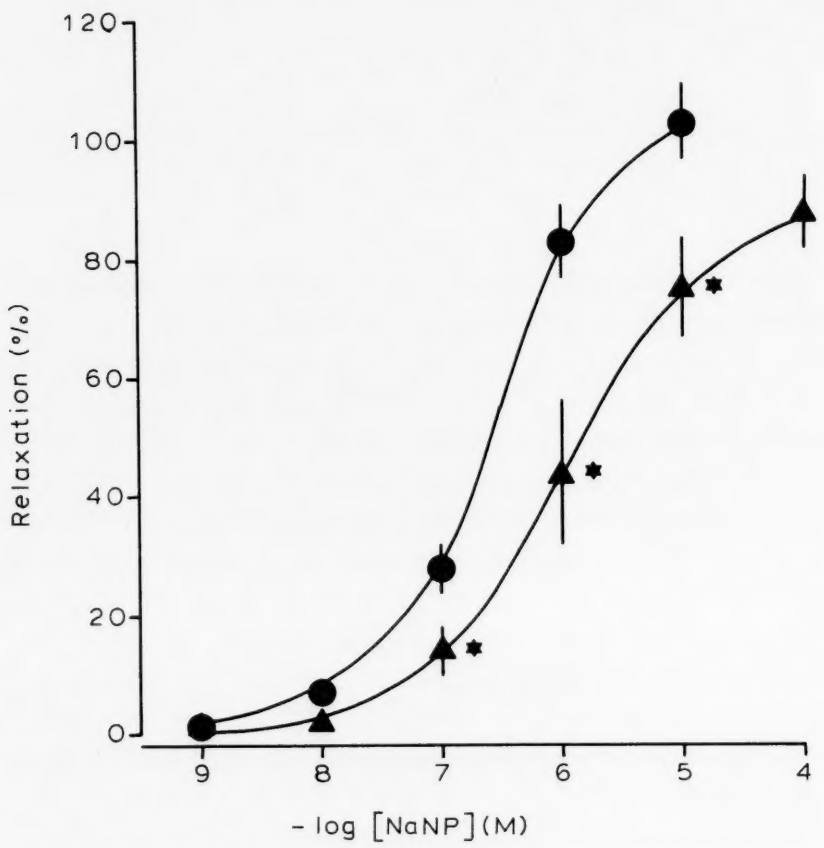

Fig. 3. Concentration-response curves showing mean ± S.E.M. % relaxation of preconstricted pig coronary artery and pig renal artery induced by sodium nitroprusside (NaNP). Relaxation responses of pig coronary artery are significantly greater than pig renal artery at all concentrations of sodium nitroprusside $> 10^{-7}$ M. (* $P < 0.05$; $n = 6$).

P appears to release more EDRF from coronary artery than from renal artery donors.

Figure 5 shows EDRF release rates (normalised for donor dimensions, flow rates and donor-recipient transit times) from pig coronary artery and pig renal artery donors in response to 60 s pulses of increasing concentrations of substance P. Basal EDRF release rates from coronary artery and renal artery endothelium were 0.93 ± 0.18 and 0.83 ± 0.15 nU/cm^2 per s, respectively. The pig coronary artery endothelium concentration response curve has a slope of 3.2 ± 0.9, an EC_{50} value of $5.9 \pm 1.6 \times 10^{-10}$ M and an asymptote of 7.8 ± 2.0 nU/cm^2 per s. The pig coronary artery endothelium concentration response curve has a slope of 0.7 ± 0.1, an EC_{50} value of $1.6 \pm 0.8 \times 10^{-9}$ M and an asymptote of 2.72 ± 0.49 nU/cm^2 per s. The EC_{50} values for the two vessels were similar but the slope and asymptote of the coronary artery endothelium concentration-re-

sponse curve is significantly greater than that for the renal artery endothelium ($P < 0.05$).

3.2.2. Bradykinin

Figure 6 shows typical tension recordings from β-blocked, endothelium-denuded pig coronary artery rings exposed to EDRF released from a pig coronary artery and pig renal artery donor. Note that bradykinin appears to release more EDRF from renal artery than from coronary artery donors.

Figure 7 shows normalised EDRF release rates from pig coronary artery and pig renal artery endothelium in response to 60 s pulses of increasing concentrations of bradykinin. Basal EDRF release rates from coronary artery and renal artery endothelium were 0.85 ± 0.18 and 0.92 ± 0.36 nU/cm^2 per s, respectively. The pig coronary artery endothelium concentration-response curve has a slope of 2.5 ± 0.7, an EC_{50} value of $3.2 \pm 0.9 \times 10^{-8}$ M and an asymptote of 9.0 ± 2.4 nU/cm^2 per s. The pig renal artery endothelium concentration response curve has a slope of 9.3 ± 3.4, an EC_{50} value of $6.0 \pm 3.1 \times 10^{-8}$ M and an asymptote of 27.4 ± 0.5 nU/cm^2 per s. The EC_{50} values of the two vessels were similar but slope and asymptote of the pig renal artery endothelium concentration-response curve is significantly greater than that for the pig coronary artery endothelium ($P < 0.05$).

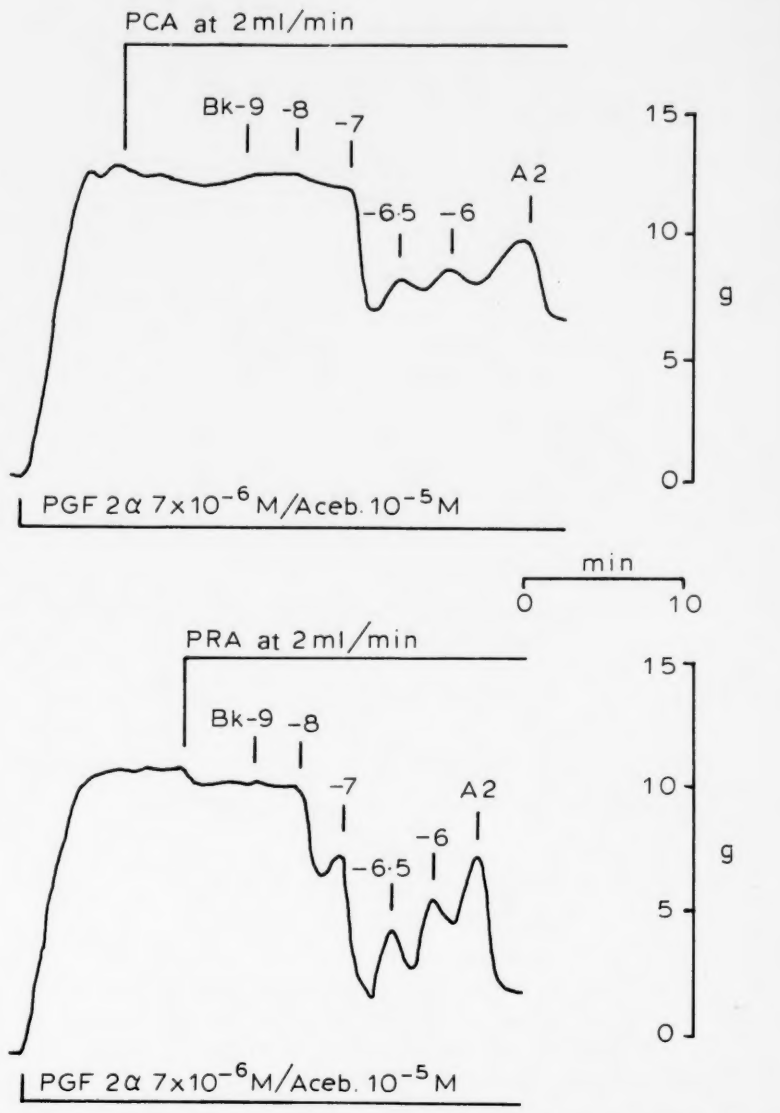

Fig. 6. Representative traces showing relaxation responses of pre-constricted endothelium-denuded pig coronary artery to EDRF released by increasing concentrations of bradykinin (BK) and a supramaximal concentration of the calcium ionophore A23187 (A2), from an endothelium-intact pig coronary artery (PCA) (upper trace) or pig renal artery (PRA) (lower trace) in a bioassay cascade. Note that relaxation responses were greater to EDRF released from the pig renal artery than pig coronary artery.

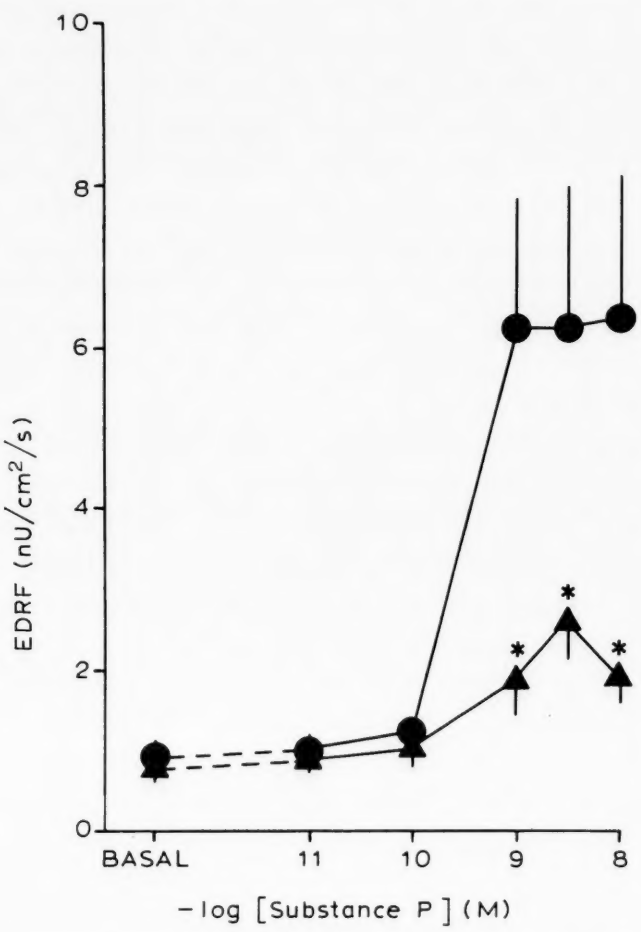

Fig. 5. Concentration-response curves showing the mean ± S.E.M. EDRF release rates from pig coronary artery and pig renal artery in unstimulated vessels (basal) and following addition of substance P in a bioassay cascade. Note that although basal EDRF release rates are similar in the two arteries, release rates in response to substance P are 2-3 times greater in pig coronary artery, at concentrations $> 10^{-10}$ M (* $P < 0.05$; $n = 5$).

4. Discussion

The results from the first part of this study indicate that differences in EDRF activity between the two vessels are due in part to differences in the sensitivity of the underlying smooth muscle to EDRF. When endothelium-denuded rings from each artery type were equilibrated at their optimum resting tension, pre-constricted to the same point on their $PGF_{2\alpha}$ concentration response curve and exposed to similar amounts of EDRF from a common pig coronary artery donor, rings of coronary artery showed a significantly greater relaxant response to EDRF. This difference was not due to the release of a 'coronary-specific' EDRF, as similar results were obtained when a pig renal artery was used as a donor. The differential response to

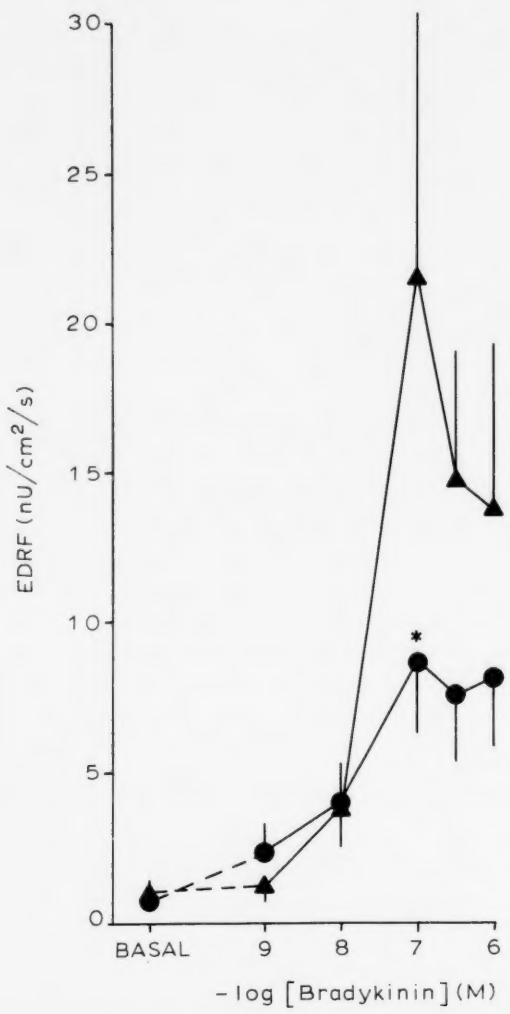

Fig. 7. Concentration-response curves showing the mean ± S.E.M. EDRF release rates from pig coronary artery and pig renal artery in unstimulated vessels (Basal) and following addition of bradykinin in a bioassay cascade. Note that although basal release rates are similar, in contrast to the findings with substance P, bradykinin induces a greater release of EDRF from pig renal artery than pig coronary artery at concentrations $> 10^{-8}$ M. (* $P < 0.05$; $n = 5$).

EDRF was parallelled by a differential response to nitroprusside and, since nitroprusside exerts its relaxant action via spontaneous release of nitric oxide (Murad et al., 1978; Gruetter et al., 1979; Feelish and Noake 1987a,b), provides further circumstantial evidence that EDRF is nitric oxide or a closely related moiety (Ignarro et al., 1987; Palmer et al., 1987).

The similarity in basal EDRF release rates between pig coronary artery and pig renal artery endothelium suggests that it is the sensitivity of the underlying smooth muscle to EDRF which is the major contributor to the degree of basal EDRF activity shown in vitro. However, the calculated shear stresses on the endothelium of the vessels used in the present bioassay system (ca. 0.1-1.0 dyn/cm^2) may be too low to activate the endothelial cells directly, as other shear-dependent endothelial processes such as prostacyclin release (Frangos et al., 1985) and hyperpolarisation (Nakache and Gaub, 1988) are reported to occur at relatively high shear stresses in excess of 10 dyn/cm^2. Recently Olesen and coworkers (1988) have reported endothelial K^+ influx responses to shear stress with half-maximal effect at 0.7 dyn/cm^2, though whether this phenomenon is related to EDRF release is not clear. Certainly in the present study there was little difference in basal EDRF release between pig coronary artery and renal artery endothelium. However, since endothelial shear stresses in the present system are lower than would be expected in vivo (due to the higher viscosity of blood and higher volume flow rates) the findings of the present study may not be directly relevant therefore to the in vivo situation.

The release of EDRF from pig coronary artery and renal artery by substance P showed a marked difference in maximal release rates, with substance P releasing EDRF from coronary endothelium at a maximum rate 2-3 times that of renal endothelium. This observation correlates well with previous in vitro studies in the dog (Angus et al., 1986a) and pig (Angus et al., 1986b), and provides a mechanism which might explain regional differences in EDRF response in terms of differential agonist-induced EDRF release. The explanation for the higher substance P-mediated EDRF release from coronary compared to renal endothelium has not been fully investigated, but is unlikely to be due to a reduced ability of the renal endothelium to synthesize or release EDRF, as renal endothelium develops a full EDRF response to the calcium ionophore A23187. Differential EDRF release in response to bradykinin is the reverse of that found to substance P, with EDRF release in response to bradykinin greater in renal than in coronary endothelium. A similar phenomenon may explain the unexpectedly high bradykinin-induced EDRF responses in dog renal artery preparations relative to their glyceryl trinitrate sensitivity previously found by Angus and colleagues (1988).

In conclusion, this study shows marked differences in the responsiveness of porcine coronary and renal arteries to EDRF and also in the ability of the endothelium of these vessels to release EDRF in response to receptor-operated agonists like substance P and bradykinin. The study draws attention to the complexity of responses possible in vitro to EDRF released by such agonists. Whether similar differences in the pattern of EDRF release are present with other physiological stimuli of EDRF release like flow, is unknown at present.

Acknowledgement

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References

Angus, J.A., T.M. Cocks and K. Satoh, 1986a, α_2-Adrenoceptors and endothelium-dependent relaxation in canine large arteries, Br. J. Pharmacol. 8, 767.

Angus, J.A., T.M. Cocks and K. Satoh, 1986b, The α-receptors on endothelial cells, Fed. Proc. 45, 2359.

Angus, J.A., T.M. Cocks, K. Satoh and T.D. White, 1988, Receptor heterogeneity and EDRF release, in: Sixth International Symposium on Vascular Neuroeffector Mechanisms, eds. J.A. Bevan, H.M. Magewski, R.A. Maxwell and D.F. Story (IRL Press Ltd.) p. 85.

Christie, M.I., T.M. Griffith and M.J. Lewis, 1989, A comparison of basal and agonist-stimulated release of endothelium-derived relaxing factor from different arteries, Br. J. Pharmacol. 98, 397.

Christie, M.I and M.J. Lewis, 1988, Vascular smooth muscle sensitivity to endothelium-derived relaxing factor is different in different arteries, Br. J. Pharmacol. 95, 630.

Collins, P., S.P. Chappell, T.M Griffith, M.J. Lewis and A.H. Henderson, 1986, Differences in basal endothelium-derived relaxing factor activity in different artery types, J. Cardiovasc. Pharmacol. 8, 1158.

Feelish, M. and E. Noake, 1987a, Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase, Eur. J. Pharmacol. 139. 19.

Feelish, M. and E. Noake, 1987b, Nitric Oxide (NO) formation from nitrovasodilators occurs independently of haemaglobin or non-heme iron, Eur J. Pharmacol. 142, 465.

Frangos, J.A., S.G. Eskin, L.V. McIntire and C.L. Ives, 1985, Flow effects on prostacyclin production by cultured human endothelial cells, Science 227, 1477.

Griffith, T.M., A.H. Henderson, D. Hughes Edwards and M.J. Lewis, 1984, Isolated perfused rabbit coronary and aortic strip preparations: the role of endothelium-derived relaxant factor, J. Physiol. 351, 13.

Gruetter, C.A., B.K. Barry, D.B. McNamara, D.J. Gruetter, P.J. Kadowitz and L.J. Ignarro, 1979, Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosamine, J. Cycl. Nucl. Res. 5, 211.

Ignarro, L.J., G.M. Buga, K.S. Wood, R.E. Byrns and G. Chaudhuri, 1987, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide, Proc. Natl. Acad. Sci. U.S.A. 84, 9265.

Murad, F., C.K. Mittal, W.P. Arnold, S. Katsuki and H. Kimura, 1978, Guanylate cyclase activation by azide, nitro compounds, nitric oxide and hydroxyl radical and inhibition by haemoglobin and myoglobin, Adv. Cycl. Nucl. Res. 9, 145.

Nakache, M. and H.E. Gaub, 1988, Hydrodynamic hyperpolarisation of endothelial cells, Proc. Natl. Acad. Sci. U.S.A. 85, 1841.

Olesen, S., D.E. Clapham and P.F. Davies, 1988, Haemodynamic shear stress activates a K^+-current in vascular endothelial cells, Nature 331, 481.

Palmer, R.M.J., D.S. Ashton and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, Nature. 327, 524.

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Pharmacological properties of cloned muscarinic receptors expressed in A9 L cells; comparison with in vitro models

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The effects of a series of muscarinic agonists and antagonists at cloned m1 and m3 muscarinic receptors expressed in mouse fibroblast A9 L cells have been compared with their effects in in vitro models of M_1 (rat superior cervical ganglion) and M_3 (guinea-pig ileum) muscarinic receptors. A good correlation existed between the potencies of muscarinic agonists at cloned ml muscarinic receptors and the M_1 sites in rat ganglion ($r = 0.80$) as well as at cloned m3 receptors and guinea-pig ileum M_3 receptors ($r = 0.87$). However, cross correlations of potencies in rat ganglion and cloned m3 receptors as well as in guinea-pig ileum and in cloned m1 receptors also yielded relatively high correlation coefficients (0.71 and 0.91, respectively). Low correlation coefficients were found for the maximal responses of muscarinic agonists in rat ganglion and cloned m1 receptors (0.53) and in guinea-pig ileum and cloned m3 receptors (0.36). A high correlation between pA_2 values of muscarinic antagonists at cloned m1 receptors and in rat ganglion ($r = 0.97$) and between cloned m3 receptors and guinea-pig ileum ($r = 0.98$) was found. Cross correlation of pA_2 values in rat ganglion and cloned m3 receptors and in guinea-pig ileum and cloned m1 receptors yielded correlation coefficients of 0.82 and 0.72, respectively. The data indicate that the cloned muscarinic receptor sites seem similar to the corresponding endogenous sites. The good correlations in corresponding but also non-corresponding receptor models reflect the relatively low selectivity of the majority of the compounds investigated.

Muscarinic receptors (cloned); A_9 L cells (mouse fibroblast); Muscarinic M_1 receptors; Muscarinic M_3 receptors

1. Introduction

Five muscarinic receptors have been cloned and expressed in various cell lines (Kubo et al., 1986; Bonner et al., 1987; Peralta et al., 1987). Biochemical studies have revealed that activation of m1, m3 and m5 muscarinic receptors expressed in A9 L cells leads to increased phosphatidylinositol (PI) metabolism. The G-protein mediating m1, m3 and m5 muscarinic receptor-induced PI metabolism is pertussis toxin-insensitive (Brann et al., 1988). Whole organ pharmacology has revealed three 'native' muscarinic receptors, M_1, M_2 and M_3 (Doods et al., 1987). Recently, a uniform nomenclature for muscarinic receptors, defined in whole organ experiments and in cloned receptors, has been devised (Birdsall et al., 1989). Activation of M_1 and M_3 muscarinic receptors in animal tissues induces an increase in PI metabolism via a pertussis toxin-insensitive G-protein (Brown et al., 1989; Horwitz et al., 1985; Salomon and Bolton, 1988). Since a clear analogy exists between the second messenger systems of M_1 and M_3 muscarinic receptors in animal tissue and those expressed in A9 L cells, it seemed of interest to compare the responses of a series of muscarinic agonists and antagonists in these two models. For this purpose the increase in free intracellular calcium induced by m1 or m3 receptor activation was measured using the calcium indicator fura-2/AM in A9 L cells. The rat superior cervical ganglion was used as an in vitro model for M_1 receptors (Brown et al., 1980) and the guinea-pig ileum was used as a model for M_3 muscarinic receptors (Lazareno and Roberts, 1989).

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2. Materials and methods

2.1. Rat superior cervical ganglion

Experiments on rat superior cervical ganglia were performed as described by Brown et al. (1980). Male rats (Sprague-Dawley 200-300 g) were anaesthetized with urethane (1.2 g/kg i.p.) and superior cervical ganglia were excised. Ganglia were desheathed, suspended vertically in a heated chamber (36°C) and

superfused (1 ml/min) with oxygenated Krebs solution of the following composition (in mM): NaCl 124.0, KCl 3.0, NaH_2PO_4, 1.25, $NaHCO_3$ 5.0, $CaCl_2$ 2.0 and glucose 10.0. The preparations were allowed to equilibrate for 30-45 min. Muscarinic agonist-induced depolarizations were recorded differentially between the ganglion and its postganglionic trunk using calomel electrodes. The DC potentials were amplified by microvoltmeters and monitored on computer. After 4-6 min perfusion with agonist, a concentration-dependent maximal depolarization was obtained which was used as a measure of agonist effect.

2.2. Guinea-pig ileum

Male guinea-pigs (300-350 g, Ivanovas) were killed by a blow to the head and a segment of the ileum was removed. A piece of longitudinal muscle strip was suspended vertically in an organ bath containing 20 m1 oxygenated Krebs solution (36°C) at a resting tension of 7.5 mN. The preparation was allowed to equilibrate for 30 min. Muscarinic agonist-induced contractions were measured with force-displacement transducers and monitored on a computer.

2.3. Measurement of free intracellular calcium

A9 L cells transfected with cloned m1 or m3 muscarinic receptors, (obtained from NIH, Bethesda, Maryland, USA) were cultured in Dulbecco's modified Eagle's Medium supplemented with 5% fetal calf serum. The cells were cultured not longer than 12 passages. Cell suspensions with a density of approximately 10^6 cells were seeded on glass coverslips and grown to confluent monolayers. The coverslips were washed in physiological salt solution (PSS; composition in mM: NaCl 145.0, KCl 5.0, $CaCl_2$ 1.9, $MgCl_2$ 1.0, HEPES 5.0 and glucose 10.0, pH 7.4). The cells were incubated at 37°C for 30 min in PSS containing 5 μM fura-2 acetylmethoxyester and 0.02% w/v pluronic. The monolayers were washed and allowed to rest for 40 min at room temperature. The glass coverslips were placed in a quartz cuvette filled with 3 ml PSS and the cuvette was placed in a thermostated cuvette-holder (37°C) of an LS-5b Perkin-Elmer Luminescence Spectrophotometer. Continuous stirring was achieved by a magnetic microstirrer. Changes in fura-2 fluorescence upon agonist stimulation of the cells were measured using an excitation wavelength of 340 nm and an emission wavelength of 509 nm. Calibration was performed with 5 μM ionomycin to determine the maximal fluorescence (F_{max}) and 5 mM $MnCl_2$ to determine minimal fluorescence (F_{min}). The value F(q) obtained after the addition of $MnCl_2$ represents the complete quenching of fura-2 fluorescence by $MnCl_2$ and has to be modified according to the following equation to yield the correct minimum fluorescence value F_{min}: $F_{min} = F(q) + 0.27 \times (F_{max} - F(q)$ (Fisher and Snider, 1987; Capponi et al., 1989).

The increase in cytosolic free calcium was calculated from the equation: $[Ca^{2+}] = K_d \times ((F - F_{min})/(F_{max} - F))$ (Grynkiewicz et al., 1985), where F is the measured fluorescence intensity and K_d is the dissociation constant of fura-2 for Ca^{2+} and has the value 225 nM. Agonist activity was defined as the maximal increase in intracellular calcium concentration induced by a single concentration of agonist.

2.4. Agonist potencies

In rat superior cervical ganglia and guinea-pig ilea, concentration-response curves were made with single concentrations (six to eight) of agonist applied at 20-45 min intervals followed by a washout phase until the baseline was reached. In the transfected cells, a complete concentration-response curve was made by using one coverslip with single concentrations (6-8) of agonist. Between the measurements the cells were allowed to rest in PSS for 15-20 min. The potency of an agonist is expressed as its pD_2 value, i.e. the $-\log$ of the molar concentration that induced a response that was 50% of the individual maximal effect. For the quantification of partial agonism, the response of carbachol was set to 100%.

2.5. Antagonist affinities

Rat superior cervical ganglia and guinea-pig ilea were allowed to equilibrate for 30-60 min. Concentration-response curves of carbachol were obtained before and after addition of an antagonist. Only one concentration-response curve per coverslip could be made with the transfected cells, Therefore, control and antagonist-shifted concentration-response curves were made with different coverslips. EC_{50} values were determined for the control and the antagonist-shifted concentration-response curves. Three to four concentrations of antagonist were tested in all preparations after a 60-min equilibration period. Each concentration of antagonist was tested 4-6 times and the ratios of agonist molar EC_{50} values obtained in the absence or presence of antagonist were calculated. Schild plots were calculated using linear regression by the method of least squares.

2.6. Statistics

All data are presented as means $\pm$ S.E.M. Statistical significance ($P < 0.05$) was evaluated by means of an analysis of variance and Student's t-test. Correlations were performed according to the method of least squares.

TABLE 1

The pD_2 values and maximal responses of a series of muscarinic agonists at cloned m1 and m3 muscarinic receptors, rat superior cervical ganglion (M_1) and guinea-pig ileum (M_3). The data represent mean values ± S.E.M. (MFMT = methylfurmethide; n.m. = not measured).

Compound	m1 receptors (pD_2)	m1 receptors (maximal response %)	M_1 receptors (pD_2)	M_1 receptors (maximal response %)
Carbachol	5.7 ± 0.1	100	7.2 ± 0.2	100
Muscarine	5.7 ± 0.1	98 ± 6.2	7.1 ± 0.1	96 ± 4.3
Oxotremorine	6.1 ± 0.3	65 ± 4.2	6.9 ± 0.1	43 ± 4.4
BM-5	6.3 ± 0.2	15 ± 2.1	7.4 ± 0.1	33 ± 4.6
Arecoline	6.0 ± 0.2	65 ± 1.2	6.6 ± 0.1	80 ± 3.8
Pilocarpine	5.5 ± 0.1	69 ± 1.4	6.2 ± 0.3	130 ± 5.9
AF-102b	4.3 ± 0.1	20 ± 3.6	6.1 ± 0.3	79 ± 6.2
AF30	4.6 ± 0.2	10 ± 2.1	6.0 ± 0.1	56 ± 4.9
RS-86	5.9 ± 0.4	58 ± 3.3	6.7 ± 0.2	80 ± 7.2
210-086	5.7 ± 0.3	49 ± 2.5	7.0 ± 0.1	136 ± 3.7
MFMT	6.0 ± 0.2	68 ± 4.6	7.2 ± 0.1	136 ± 9.2
McN-A-343	5.1 ± 0.2	56 ± 8.1	6.0 ± 0.2	122 ± 7.3
Compounds	**m3 receptors (pD_2)**	**m3 receptors (maximal response %)**	**M_3 receptors (pD_2)**	**M_3 receptors (maximal response %)**
Carbachol	5.6 ± 0.4	100	6.7 ± 0.3	100
Muscarine	5.6 ± 0.1	104 ± 9.2	6.8 ± 0.1	95 ± 2.7
Oxotremorine	6.6 ± 0.8	53 ± 2.2	7.1 ± 0.1	92 ± 4.9
BM-5	6.2 ± 0.9	10 ± 1.4	6.6 ± 0.2	107 ± 7.1
Arecoline	6.1 ± 0.6	32 ± 1.6	6.5 ± 0.1	109 ± 3.5
Pilocarpine	5.3 ± 1.0	5 ± 0.8	5.9 ± 0.2	84 ± 4.3
AF-102b	4.5 ± 0.7	10 ± 2.7	4.9 ± 0.1	52 ± 2.9
AF-30	4.7 ± 0.6	21 ± 4.6	4.8 ± 0.2	91 ± 4.7
RS86	6.2 ± 0.5	29 ± 1.1	6.1 ± 0.4	97 ± 8.2
210-086	5.7 ± 0.4	58 ± 2.1	6.3 ± 0.1	111 ± 7.6
MFMT	6.2 ± 0.5	61 ± 3.3	6.8 ± 0.3	115 ± 6.9
McN-A-343	< 4.0	n.m.	< 4.0	n.m.

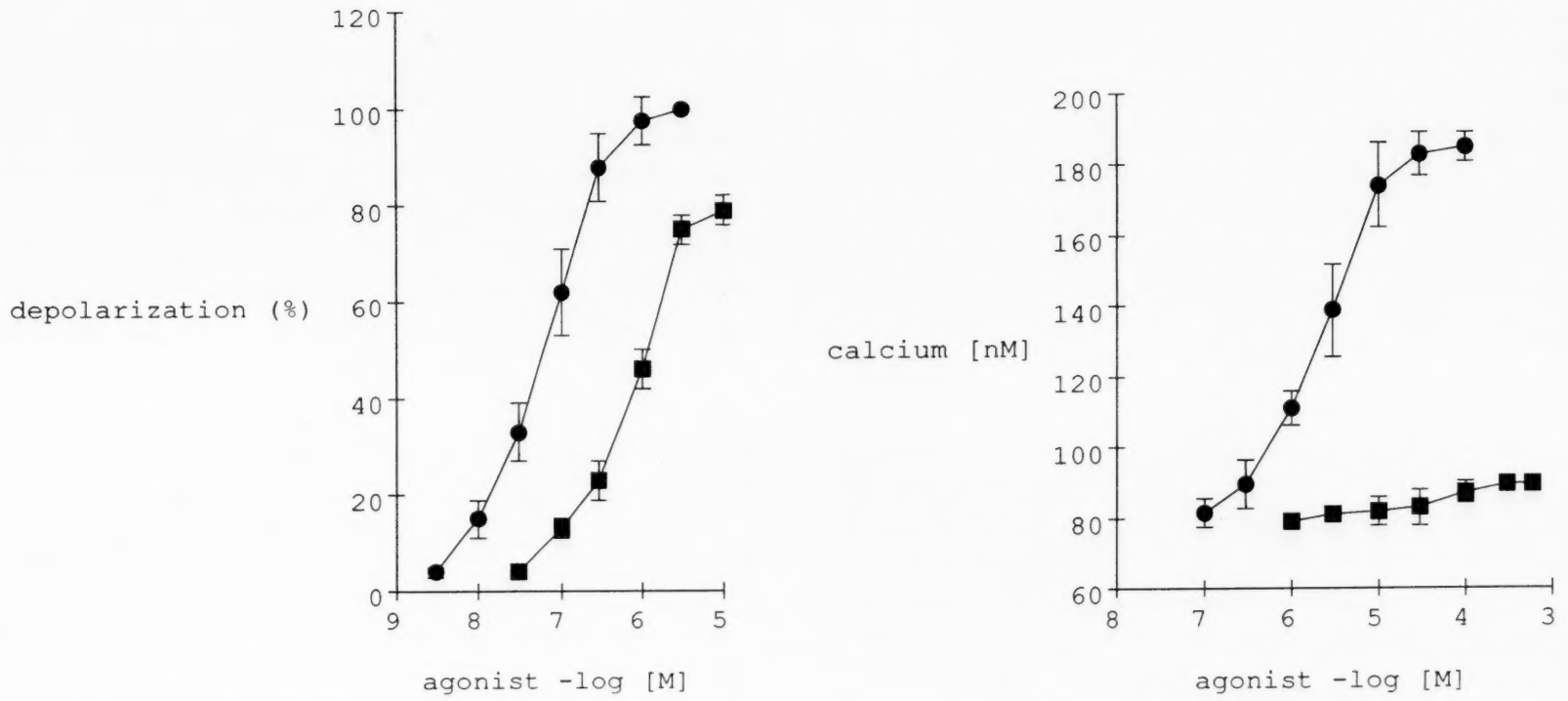

Fig 1. (A) The concentration-response curve for the depolarization in rat superior cervical ganglion induced by carbachol (●) and AF-102b (■). (B) The concentration-response curve for the contraction in guinea-pig ileum induced by carbachol (●) and AF-102b (■). The data represent mean values ± S.E.M. (n = 6-8).

2.7. Compounds used

Carbamoylcholine hydrochloride, E. Merck, Darmstadt, FRG; DL-muscarine chloride, Sigma St. Louis MO, USA; oxotremorine sesquifumarate, Sigma St. Louis MO, USA; BM-5 (N-methyl-N-(1-methyl-4-pyrrolidino-1-yl-2-butynyl)acetamide oxalate), gift Professor D. Jenden, UCLA University, USA; arecoline hydrobromide, Fluka AG, Buchs, Switzerland; pilocarpine hydrochloride, Sigma St. Louis MO, USA; AF-102b (2-methyl-spiro(1,3-oxothiolan-4,3)-quinuclidine), Sandoz Ltd., Switzerland; AF-30 (2-methyl-spiro (1,3-dioxolan-4,3)-quiniclidine), Sandoz Ltd., Switzerland; RS86 (2-ethyl-8-methyl-2,8-diazaspiro-(4,5)-decan-1,3-dion hydrobromide), Sandoz Ltd., Switzerland; 210-086 ((−)[2-methyl-spiro-(1,3-dioxolan-4,4]-1-methylpiperidine hydrogenmaleinate), Sandoz Ltd., Switzerland; methylfurmethide (5-methylfurfuryltrimethyl ammonium iodide), Sandoz Ltd., Switzerland; McN-A-343 (4-(3-chlorophenylcarbamoyloxy)-2-butinyltrimethyl ammonium chloride), RBI, Natick, MA, USA; atropine methylnitrate, Sigma, St. Louis MO, USA; pirenzepine, gift Boehringer Ingelheim, FRG; telenzepine dihydrochloride, gift Byk Gulden, D-Lomberg, FRG; AF-DX 116, gift Boehringer Ingelheim, FRG; methoctramine tetrahydrochloride, RBI, Natick MA, USA; para-fluoro-hexahydro-sila-difenidol hydrochloride, gift Professor G. Lambrecht, University of Frankfurt, FRG; 4-DAMP methiodide, RBI, Natick MA, USA; fura-2/acetylmethoxy ester, Sigma, St. Louis MO, USA; pluronic, Sigma, St. Louis MO, USA; ionomycin, Serva, Heidelberg, FRG.

All drugs were dissolved in a saline (0.9%) solution. Stock solutions of fura-2 and ionomycin were prepared in DMSO; the final concentration of DMSO did not exceed 0.1%.

3. Results

3.1. Rat superior cervical ganglion

The 12 agonists studied all induced a concentration-dependent depolarization of rat superior cervical ganglia. The corresponding pD_2 values are listed in table 1. Full agonist behaviour was observed for carbachol, which induced a maximal depolarization of 0.92 $\pm$ 0.08 mV (fig. 1), muscarine, pilocarpine, methylfurmethide, 210-086 and McN-A-343. The other compounds behaved as partial agonists, with maximal responses varying from 33 $\pm$ 4.6 to 80 $\pm$ 3.7% compared with carbachol (table 1). All antagonists investigated produced parallel shifts of the concentration-response curve of carbachol, as indicated by Schild analysis yielding slopes not significantly different from 1. The pA_2 values are listed in table 2.

3.2. Guinea-pig ileum

In fig. 1 the concentration-response curve of carbachol the guinea-pig ileum is shown. The maximal contraction induced by carbachol (43 $\pm$ 4.2 mN) was set to 100%. With the exception of McN-A-343, all the muscarinic agonists studied contracted guinea-pig ileum in a concentration-dependent manner (table 1). Pilocarpine and AF-102b behaved as partial agonists, with maximal responses of 84 $\pm$ 5.6 and 52 $\pm$ 3.4% of the maximal response of muscarine, respectively. All an-

TABLE 2

The pA_2 values and the slopes of the Schild plots of a series of muscarinic antagonists at cloned m1 and m3 muscarinic receptors, rat superior cervical ganglion (M_1) and guinea-pig ileum (M_3). The data represent mean values $\pm$ S.E.M.

Compound	m1 receptors (pA_2)	m1 receptors (slope)	M_1 receptors (pA_2)	M_1 receptors (slope)
Atropine	8.6 $\pm$ 0.10	1.08 $\pm$ 0.21	9.1 $\pm$ 0.13	0.98 $\pm$ 0.11
Pirenzepine	8.1 $\pm$ 0.07	0.86 $\pm$ 0.16	8.3 $\pm$ 0.09	0.96 $\pm$ 0.14
Telenzepine	8.3 $\pm$ 0.24	0.93 $\pm$ 0.09	8.4 $\pm$ 0.11	0.84 $\pm$ 0.13
AF-DX 116	6.4 $\pm$ 0.18	0.89 $\pm$ 0.06	6.3 $\pm$ 0.17	1.05 $\pm$ 0.08
Methoctramine	7.6 $\pm$ 0.14	0.96 $\pm$ 0.13	7.3 $\pm$ 0.08	0.94 $\pm$ 0.04
pF-HHSiD	7.1 $\pm$ 0.05	1.07 $\pm$ 0.09	7.2 $\pm$ 0.04	0.88 $\pm$ 0.07
4-DAMP	9.1 $\pm$ 0.08	0.95 $\pm$ 0.04	9.0 $\pm$ 0.14	0.91 $\pm$ 0.11
Compound	m3 receptors (pA_2)	m3 receptors (slope)	M_3 receptors (pA_2)	M_3 receptors (slope)
Atropine	8.9 $\pm$ 0.16	0.87 $\pm$ 0.06	8.9 $\pm$ 0.09	1.06 $\pm$ 0.07
Pirenzepine	7.0 $\pm$ 0.06	0.92 $\pm$ 0.11	6.8 $\pm$ 0.13	0.94 $\pm$ 0.10
Telenzepine	7.1 $\pm$ 0.08	1.09 $\pm$ 0.08	6.7 $\pm$ 0.05	1.02 $\pm$ 0.08
AF-FX 116	6.1 $\pm$ 0.13	0.96 $\pm$ 0.10	6.1 $\pm$ 0.07	0.89 $\pm$ 0.11
Methoctramine	6.9 $\pm$ 0.16	1.11 $\pm$ 0.05	6.5 $\pm$ 0.08	1.07 $\pm$ 0.04
pF-HHSiD	7.6 $\pm$ 0.09	1.17 $\pm$ 0.12	7.7 $\pm$ 0.09	1.01 $\pm$ 0.03
4-DAMP	8.9 $\pm$ 0.14	0.86 $\pm$ 0.08	9.5 $\pm$ 0.10	1.12 $\pm$ 0.09

TABLE 3

The correlations of the potencies, the maximal responses and the antagonist affinities of muscarinic agonists and antagonists at cloned m1 and m3 muscarinic receptors, rat superior cervical ganglion (M_1) and guinea-pig ileum (M_3).

	Correlations	
	Correlation coefficient (r)	Regression equation
Agonist potencies		
Cloned m1 vs. in vitro M_1	0.80	$Y = 0.67X + 2.97$
Cloned m3 vs. in vitro M_3	0.87	$Y = 1.00X + 0.51$
Cloned m1 vs. in vitro M_3	0.91	$Y = 1.11X + 0.003$
Cloned m3 vs. in vitro M_1	0.71	$Y = 0.52X + 3.78$
In vitro M_1 vs. in vitro M_3	0.85	$Y = 1.47X + 3.81$
Maximal responses		
Cloned m1 vs. in vitro M_1	0.53	$Y = 0.60X + 52.54$
Cloned m3 vs. in vitro M_3	0.36	$Y = 0.18X + 87.72$
Antagonist affinities		
Cloned m1 vs. in vitro M_1	0.97	$Y = 1.09X - 0.68$
Cloned m3 vs. in vitro M_3	0.98	$Y = 1.20X - 1.56$
Cloned m1 vs. in vitro M_3	0.72	$Y = 1.00X - 0.50$
Cloned m3 vs. in vitro M_1	0.82	$Y = 0.80X + 1.91$
In vitro M_1 vs. in vitro M_3	0.73	$Y = 0.92X + 0.18$

tagonists studied shifted the concentration-response curve of carbachol in a parallel manner. The pA_2 values and the slopes of the Schild plots are given in table 2.

3.3. Muscarinic m1 and m3 receptors in A9 L cells

Concentration-dependent increases in cytosolic calcium were induced by addition of muscarinic agonists in A9 L cells transfected with either m1 or m3 muscarinic receptors. Concentration-response curves of carbachol at both m1 and m3 receptors are shown in fig. 2. Carbachol and muscarine showed full agonist behaviour at both m1 and m3 receptors and induced maximal increases in intracellular calcium of 190 ± 3 and 185 ± 5 nM, respectively. Whereas McN-A-343 was a partial agonist at m1 receptors, no activity for this compound could be detected at m3 receptors. Partial agonist behaviour at m1 and m3 receptors was also found for the other muscarinic agonists studied. The pD_2 values and the maximal responses for all agonists investigated are given in table 1. All antagonists investigated shifted the concentration-response curve of carbachol for its response at either m1 or m3 receptors to the right in a parallel fashion and no depression of the maxima occurred. At both m1 and m3 receptors the slopes of the Schild plots were not significantly different from unity. The pA_2 values of the antagonists investigated are given in table 2.

3.4. Correlation of in vitro systems and cloned muscarinic receptors

All correlation coefficients and the corresponding regression equations are presented in table 3. In rat superior cervical ganglion and cloned m1 receptors, a good correlation between the potencies of muscarinic agonists ($r = 0.80$) was found. A lower correlation coefficient ($r = 0.53$) was observed for the maximal responses of the muscarinic agonists in both systems. Correlation coefficients of 0.87 and 0.36 were found for pD_2 values and maximal responses, respectively, at cloned m3 muscarinic receptors and guinea-pig ileum. Correlation of the pD_2 values at ganglion and cloned

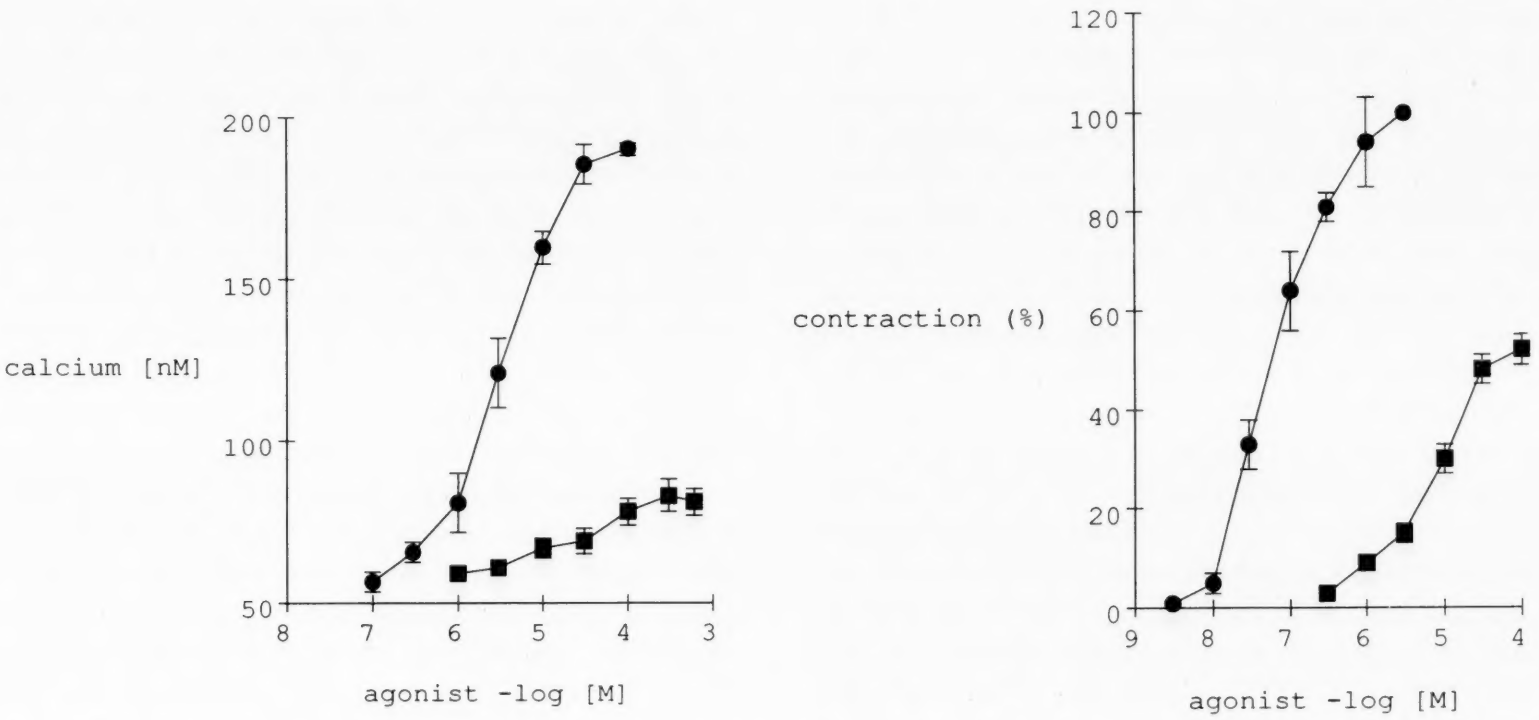

Fig 2. (A) The concentration-response curve for the increase in intracellular calcium induced by carbachol (●) and AF-102b in A9 L cells transfected with ml (■) muscarinic receptors. (B) The concentration-response curve for the increase in intracellular calcium induced by carbachol (●) and AF-102b in A9 L cells transfected with m3 (■) muscarinic receptors. The data represent mean values ± S.E.M. (n = 6-8).

m3 receptors or at ileum and cloned m1 receptors yielded correlation coefficients of 0.71 and 0.87, respectively. In addition, a good correlation between potencies in rat superior cervical ganglion and guinea-pig ileum ($r = 0.85$) was found. Excellent correlations ($r = 0.97$ and 0.98) were found for the pA_2 values of a series of muscarinic antagonists at rat superior cervical ganglion and cloned m1 receptors and at guinea-pig ileum and cloned m3 receptors, respectively. Cross correlations of pA_2 values at rat superior cervical ganglion and cloned m3 receptors or guinea-pig ileum and cloned m1 receptors yielded lower correlation coefficients of 0.82 and 0.72, respectively. Also a lower correlation coefficient was found ($r = 0.73$) for the correlation of pA_2 values in rat superior cervical ganglion and guinea-pig ileum.

4. Discussion

Recently, five subtypes of muscarinic receptors have been cloned and expressed in an A9 L mouse fibroblast cell line (Bonner, 1989). Activation of cloned m1, m3 and m5 receptors in these cells induces an increase in PI turnover (Brann et al., 1988). An increase in phosphoinositide hydrolysis and thus generation of the second messenger IP_3 causes a rapid transient release of intracellular calcium in the cytoplasm of cells (Berridge, 1977). Indeed, an increase in free cytosolic calcium and calcium-dependent increases of potassium and chloride conductances have been demonstrated in A9 L cells after stimulation of m1 or m3 muscarinic receptors (Jones et al., 1990). In the present study we describe a method which allows the activity of muscarinic compounds to be defined in these transfected A9 L cells in an easy and efficient manner. Well-attached monolayers of cells were cultured on glass coverslips and could be used several times to quantify agonist-induced changes in intracellular calcium. No desensitization was observed when up to six concentrations of muscarinic agonist were tested on one coverslip (data not shown). This enabled us to make a complete concentration-response curve with one monolayer of cells. Thus A9 L cells provide the basis for an efficient measurement of intracellular calcium. In order to establish the validity of this new test system, the pharmacological characteristics of the transfected cells were compared with those in vitro. As a model for M_1 muscarinic receptors, the slow depolarization evoked in rat superior cervical ganglion was used (Brown et al., 1980). An increase in PI turnover is believed to play an important role in this depolarization (Brown et al., 1989, Patterson and Volle, 1984). For muscarinic M_3 receptor activation, the contractile response in guinea-pig ileum (Lazareno and Roberts, 1989), which is also mediated by an increase in PI turnover, was studied.

The affinities of the antagonists compared well with those from radioligand binding studies in similarly transfected cells (Buckley et al., 1989), which supports the use of transfected cell lines as described in this study. Moreover, the data found in the transfected cells, both for agonists and antagonists, are generally consistent with those found in rat superior cervical ganglion and guinea-pig ileum described in this study. Whereas agonist potencies as well as antagonist affinities in the transfected cell lines correlated well with the corresponding in vitro systems, relatively good correlations were also observed for the non-corresponding (m1 vs. M_3 and m3 vs. M_1) systems (see table 3). This indicates that most of the compounds investigated lack receptor subtype selectivity. Nevertheless, selective antagonists like pirenzepine and telenzepine, as well as the agonist McN-A-343, displayed selectivity both in transfected cells and in vitro. For instance, the M_1 selectivity of McN-A-343 found when using rat superior cervical ganglion and guinea-pig ileum was also observed in transfected cells.

AF-102b and AF-30 were the only two agonists of which the results in animal tissue and at cloned muscarinic receptors were inconsistent. Whereas these compounds show a preference for M_1 over M_3 receptors in the in vitro models, no selectivity at the cloned muscarinic receptors was observed (see table 1). One possibility might be that the activity of both AF-102b and AF-30 does not reflect muscarinic receptor activation. However, this seems unlikely since the responses of the two agonists in rat superior cervical ganglion and guinea-pig ileum could be blocked completely by atropine (10^{-6} M) (data not shown). At present we have no explanation for the lack of selectivity of AF-102b and AF-30 at the cloned muscarinic receptors.

The potencies of the muscarinic agonists found in the cells transfected with m1 muscarinic receptors were 4- to 63-fold lower than those found in rat superior cervical ganglion (table 1); 1.3- to 16-fold lower potencies at m3 receptors than in guinea-pig ileum were found. The potency of an agonist depends on the number of steps and thus amplification between receptor activation and the parameters measured (Kenakin, 1984). Thus the different parameters measured, i.e. calcium mobilization vs. depolarization and calcium mobilization vs. contraction, may be a factor in the potency differences of the agonists found in the different systems. Moreover, it may well be that differences in the type and number of G-proteins in the various tissues exist which possibly affect agonist-receptor coupling. In addition, differences in receptor reserve may determine differences in agonist potencies. Some of the compounds that were full agonists in the in vitro models were partial agonists in the transfected cells, again indicating differences in receptor reserve (table 1). Differences in receptor reserve would be expected

to underlie the relatively weak correlations obtained between the maximal responses in the cloned receptor systems and the in vitro models. A feature which may complicate the interpretation of agonist data determined in the rat superior cervical ganglion is the M_2 receptor-mediated hyperpolarization (Newberry et al., 1985; Newberry and Gilbert, 1989). This may explain the observation that compounds like pilocarpine, 210-086, methylfurmethide and McN-A-343 produced higher maximal responses compared to carbachol in the rat superior cervical ganglion whereas they behaved as partial agonists in the cells transfected with m1 receptors. The good correlation found between the pD_2 values in the rat superior cervical ganglion and the A9 L cells transfected with m1 receptors, however, suggests that this is not an important factor. In theory, cell lines expressing only one receptor subtype, as is the case in A9 L cells transfected with cloned muscarinic receptors, would be expected to detect the activity and selectivity of muscarinic drugs more reliably than animal tissues, which generally contain more than one muscarinic receptor subtype. The use of transfected cells as described in this paper provides an efficient method to determine potencies and affinities of muscarinic agonists and antagonists, respectively, and provides data generally consistent with that of existing in vitro models. The lack of well discriminating muscarinic ligands, however, makes it difficult to estimate the predicative value of cloned receptor systems for in vitro models.

References

Berridge, M.J., 1987, Inositol triphosphate and diacylglycerol: two interacting second messengers, Ann. Rev. Biochem. 56, 159.

Birdsall, N.J.M., N. Buckley, H.N. Doods, K. Fukuda, R. Giachetti, R. Hammer, H. Kilbinger, G. Lambrecht, E. Mutschler, N. Nathanson, A. North and R. Schwarz, 1989, Nomenclature for muscarinic receptors recommended by symposium, Trends Pharmacol. Sci. 10 (Suppl. Subtypes of Muscarinic Receptors IV), VII.

Brann, M.R., B. Conklin, N.M. Dean, R.M. Collins, T.I. Bonner and N.J. Buckley, 1988, Cloned muscarinic receptors couple to different G-proteins and second messengers, Soc. Neurosci. Abstr. 14, 600.

Brown, D.A., A. Forward and S. Marsh, 1980, Antagonist discrimination between ganglionic and ileal muscarinic receptors, Br. J. Pharmacol. 71, 362.

Brown, D.A., N.V. Marrion and T.G. Smart, 1989, On the transduction mechanism for muscarine-induced inhibition of M-current in cultured rat sympathetic neurones, J. Physiol. 413, 469.

Bonner, T.I., 1989, The molecular basis of muscarinic receptor diversity, Trends Neurosci. 12, 148.

Bonner, T.I., A.C. Young, M.R. Brann and N.J. Buckley, 1987, Cloning and expression of the human and rat m5 muscarinic receptor genes, Neuron 1, 403.

Buckley, N.J., T.I. Bonner, C.M. Buckley and M.R. Brann, 1989, Antagonist binding properties of five cloned muscarinic receptors in CHO-K1 cells, Mol. Pharmacol. 35, 469.

Capponi, A.M., P.D. Lew, W. Schlegel and T. Pozzan, 1989, Use of intracellular calcium and membrane potential fluorescent indicators in neuroendocrine cells, in: Neuroendocrine Peptide Methodology, Selected Methods in Enzymology, ed. P.M. Conn (Academic Press, New York) p. 315.

Doods H.N., M.N. Mathy, D. Davidesko, K.J. Van Charldorp, A. De Jonge and P.A. Van Zwieten, 1987, Selectivity of muscarinic agonists in radioligand and in vitro experiments for the putative M_1, M_2 and M_3 receptors, J. Pharmacol. Exp. Ther. 242, 257.

Fisher, S.K. and R.H. Snider, 1987, Differential receptor occupancy requirements for muscarinic cholinergic stimulation of inositol lipid hydrolysis in brain and in neuroblastomas, Mol. Pharmacol. 32, 81.

Grynkiewiecz, G., M. Poenie and R.Y. Tsien, 1985, A new generation of calcium indicators with greatly improved fluorescence properties, J. Biol. Chem. 260, 3440.

Horwitz, J., S. Tsymbalow and R.L. Perlman, 1985, Muscarine stimulates the hydrolysis of inositol-containing phospholipids in the superior cervical ganglion, J. Pharmacol. Exp. Ther. 233, 235.

Jones, S.V.P., J.L. Barker, M.B. Goodman and M.R. Brann, 1990, Inositol triphosphate mediates cloned muscarinic receptor-activated conductances in transfected mouse fibroblast A9 L cells, J. Physiol. 412, 499.

Kenakin, T.P., 1984, The classification of drugs and drug receptors in isolated tissues, Pharmacol. Rev. 63, 165.

Kubo, T., K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishima, T. Haga, M. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose and S. Numa, 1986, Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor, Nature 323, 411.

Lazareno, S. and F.F. Roberts, 1989, Functional and binding studies with M_2-subtype selective antagonists, Br. J. Pharmacol. 98, 309.

Newberry, N.R. and M.J. Gilbert, 1989, Biphasic dose response curve to muscarine on the rat superior cervical ganglion, Eur. J. Pharmacol. 163, 237.

Newberry, N.R., T. Priestley and G.N. Woodruff, 1985, Pharmacological distinction between two muscarinic responses on the isolated superior cervical ganglion of the rat, Eur. J. Pharmacol. 116, 191.

Patterson, B.A. and R.L. Volle, 1984, Muscarinic receptors and [^{3}H]inositol incorporation in a rat sympathetic ganglion cell, J. Auton. Nerv. Syst. 10, 69.

Peralta E.G., A. Ashkenazi, J.W. Winslow, D.H. Smith, J. Ramachandran and D.J. Capon, 1987, Distinct primary structures, ligand binding and tissue specific expression of four human muscarinic acetylcholine receptors, EMBO J. 6, 3923.

Salomon, D.M.W. and T.B. Bolton, 1988, Early events in inositol phosphate metabolism in longitudinal smooth muscle from guinea-pig intestine stimulated with carbachol, Biochem. J. 245, 553.

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Behavioral effects of benzodiazepine ligands in non-dependent, diazepam-dependent and diazepam-withdrawn baboons

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Acute i.m. injections of benzodiazepine receptor ligands were administered to baboons before 1-h observational sessions. The agonist midazolam produced sedative effects, the antagonist flumazenil produced no behavioral effects, the inverse agonist FG7142 produced tremor and the inverse agonist 3-carboethoxy-β-carboline hydrochloride (βCCE) produced tremor, vomiting, jerks and seizures. Co-administration of these drugs (midazolam + βCCE, midazolam + flumazenil or flumazenil + βCCE) produced a mutual antagonism of these effects. Compared to the non-dependent condition, in the diazepam-dependent condition (baboons maintained on 20 mg/kg per day diazepam) and withdrawn condition (dependent baboons tested during withdrawal), midazolam produced decreased sedative effects, flumazenil produced increased effects (i.e., tremor, vomiting and jerks), and βCCE produced increased frequency of seizures. Taken together, these data suggest that (1) benzodiazepine receptor ligands lie on a continuum of behavioral activity, and (2) chronic diazepam administration alters the behavioral effects of these benzodiazepine ligands, producing a shift in the direction of the inverse agonist.

Diazepam; β-CCE (3-carboethoxy-β-carboline hydrochloride); FG7142; Flumazenil; Midazolam; Dependence; GABA (γ-aminobutyric acid); (Baboon)

1. Introduction

The discovery of the pharmacologically unique GABA/benzodiazepine receptor complex that mediates bidirectional effects of different drugs has led to new concepts of receptor functioning and drug action. The benzodiazepine receptor is thought to be a modulatory site coupled to the $GABA_A$ receptor-chloride channel that functions as a secondary receptor affecting ligand binding and/or channel gating of the primary GABA receptor (Haefely, 1990). Three classes of receptor ligands (agonists, antagonists and inverse agonists) that bind with high affinity to the GABA/benzodiazepine receptor system have been shown to differentially modulate the effects mediated by GABA, producing a spectrum of biochemical and behavioral effects (cf. Richards et al., 1986; Braestrup et al., 1983; Polc et al., 1982) Within this theoretical framework, benzodiazepine receptor agonists can be considered to have positive intrinsic activity: they have anticonvulsant, anxiolytic and sedative properties, and enhance the effects of GABA (γ-aminobutyric acid). Inverse agonists have negative intrinsic activity: they produce effects that are mirror images of those produced by the benzodiazepine agonists (e.g. anxiogenic and convulsant and reduce the effects of GABA in vivo). Antagonists have little or no intrinsic activity: they bind to the receptor and block the receptor-mediated behavioral and pharmacological effects of both agonists and inverse agonists.

Chronic administration of benzodiazepine agonists may produce functional changes at the benzodiazepine receptor that result in alterations in the behavioral effects of the benzodiazepine receptor ligands. It is well established that chronic treatment with benzodiazepine agonists results in tolerance to the sedative or anticonvulsant effects of the drug (File, 1985; Gonsalves and Gallager, 1985, 1987; Petersen and Jensen, 1987a, b; Tietz and Rosenberg, 1988; Sannerud et al., 1989) and changes in GABA functioning (Gonsalves and Gallager, 1985, 1987; Lösher and Stephens, 1988; Tietz et al., 1989). In addition, chronic exposure to benzodiazepine agonists have also been reported to alter the effects of benzodiazepine antagonists and

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inverse agonists (Petersen and Jensen, 1987a, b; Little et al., 1987; Takada et al., 1989). For example, previously behaviorally inactive benzodiazepine receptor antagonists provoke a withdrawal syndrome (including signs of vomiting, abnormal postures and seizures) in non-human primates after a period of chronic agonist administration (Cumin et al., 1982; Lukas and Griffiths, 1982, 1984; Lamb and Griffiths, 1984). Studies in mice have shown an increased sensitivity to both the effects of antagonists and inverse agonists after chronic agonist treatments, suggesting a shift in efficacy (termed 'withdrawal shift') along a continuum of activity in the direction of the inverse agonist (Little et al., 1987).

Benzodiazepine receptor ligands can be useful tools for analyzing the bidirectional effects mediated at the GABA/benzodiazepine receptor complex and can be used to help elucidate the mechanisms of receptor alterations that occur during chronic agonist administration. The present study was undertaken to investigate the behavioral effects of a benzodiazepine agonist, an antagonist, and two inverse agonists in non-human primates using observational methods previously shown sensitive to the effects of these compounds. One set of experiments characterized the effects of acute administration of several benzodiazepine ligands alone and in combination in non-dependent baboons. The compounds investigated were the agonist midazolam, the antagonist flumazenil, the inverse agonists FG7142 and 3-carboethoxy-β-carboline hydrochloride (βCCE); interactions among midazolam, flumazenil and βCCE were also studied. A second set of experiments assessed the effects of acute administration of most of these same benzodiazepine ligands, alone and in combination, in diazepam-dependent baboons. More specifically, several experiments characterized the effects of the agonist midazolam, the antagonist flumazenil, the inverse agonist βCCE and the interactions of midazolam or flumazenil in combination with βCCE in diazepam dependent baboons. Other experiments characterized the effects of acute administration of flumazenil and βCCE in baboons withdrawn from diazepam.

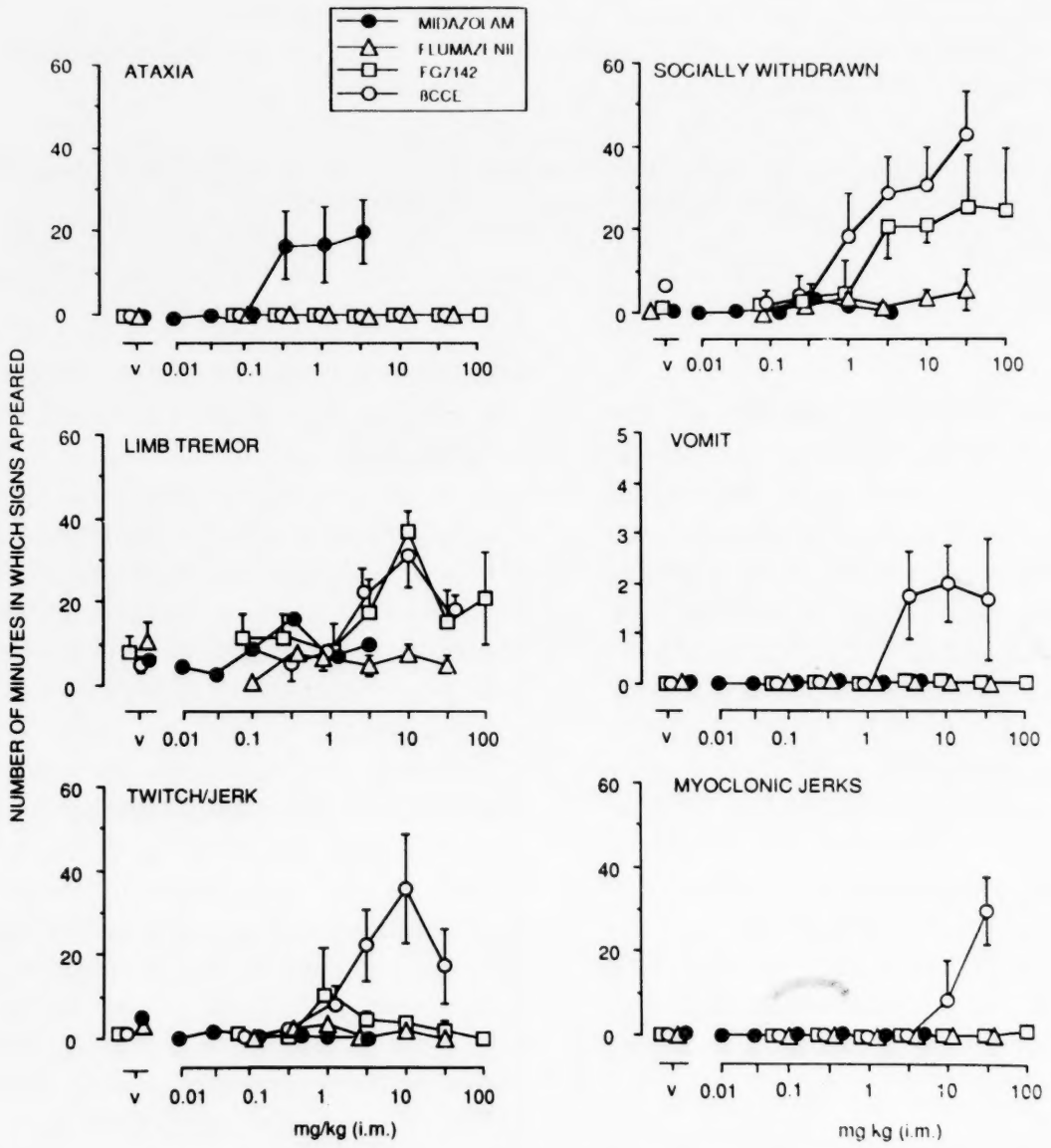

Fig. 1. Behavioral effects produced by midazolam, flumazenil, FG7142 and βCCE in the non-dependent condition. Behavioral signs were scored continuously during a 1-h observational session after i.m. administration of vehicle or drug. X axes: drug dose, log scale; vehicle (V). Y axes: the number of min during the 60-min observational session in which the signs appeared. Data points show means (n = 3-5). Brackets indicate 1 S.E.; upper or lower bracket has sometimes been deleted for clarity.

2. Materials and methods

2.1. Subjects

Eight male baboons (*Papio cynocephalus* of anubis and cynocephalus types; Primate Imports, NY, NY) weighing 19-37 kg were used as subjects in one or more of the three experimental conditions: non-dependent condition, dependent condition or withdrawn condition. Non-dependent baboons (BE, MI, TH, OP, AR, OM) were not catheterized. Dependent baboons (CH, NU, OM, AR, BE, MI, TH) and withdrawn baboons (CH, NU, BE, MI, TH) were surgically implanted with intragastric (i.g.) catheters (Lukas et al., 1982) and maintained on 20 mg/kg per day i.g. diazepam. Immediately before the experiment, these baboons had been continuously maintained on diazepam for 3 months to 1 year. These baboons were tolerant to the behavioral effects of diazepam; there were no behavioral signs of sedation prior to the start of these conditions. In the withdrawn condition, vehicle was substituted for diazepam for 4 days prior to testing.

For those baboons which served as subjects in multiple conditions, the sequence of conditions varied across subjects. Baboons BE, MI, TH served as subjects sequentially in the non-dependent condition, dependent condition, and withdrawn condition. Baboons AR and OM completed the dependent condition and, after 4 months of being drug free, were tested in the non-dependent condition. Baboons CH and NU completed the dependent condition and then were tested in the withdrawn condition.

Baboons had continuous ad libitum access to water via a drinking tube and to monkey biscuits (Purina Monkey Chow or Charles River Biscuits). Baboons received two pieces of fresh produce and a multivitamin daily.

2.2. Apparatus

Baboons were housed in standard stainless steel primate cages with a bench running the length of one wall. Each cage, which served as the experimental chamber, was surrounded by a sound attenuating dou-

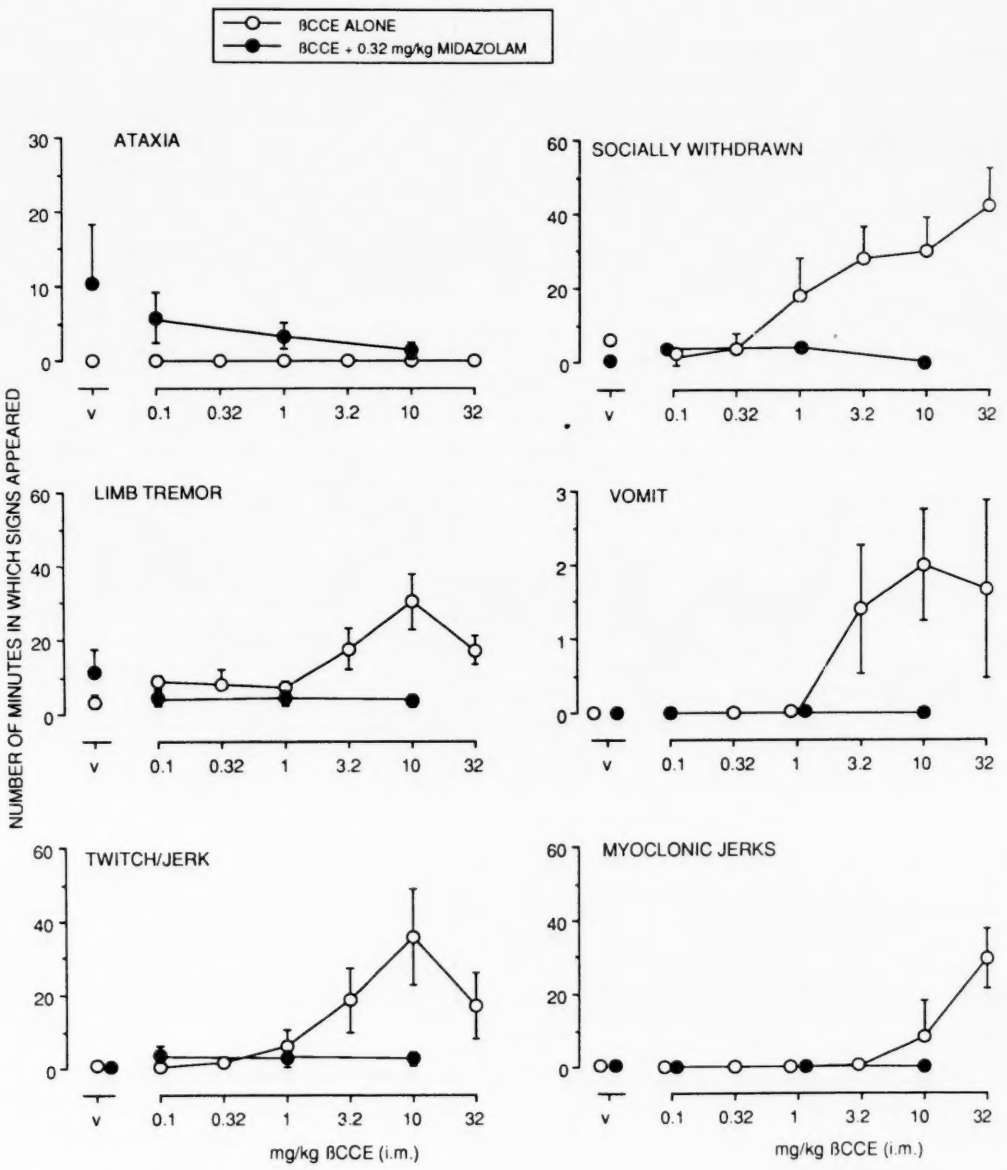

Fig. 2. Behavioral effects of βCCE alone (n = 5) and βCCE in combination with 0.32 mg/kg midazolam (n = 4) in non-dependent baboons. Dose-effect functions for βCCE alone are redrawn from fig. 1. Details as in fig. 1.

ble walled plywood external enclosure and was continuously illuminated with a 20-W frosted bulb.

2.3. Behavioral observations

The behavioral effects of benzodiazepine receptor ligands were assessed in baboons using the behavioral rating scale previously described by Sannerud et al. (1989). On test days, an acute dose of drug was administered to an individual baboon immediately before a 1-h observational session. The observational session permitted the scoring of a wide variety of behaviors, postures, movements and position in cage, and the assessment of coordination, level of activity, sedation and tremor. During the 1-h observational session, behavior was recorded continuously, and the passage of time was recorded at 1-min intervals. The presence or absence of signs in each 1-min block was tabulated. Thus, the maximum score for each sign in a 1-h observation period was 60. Observations were performed by observers blind to the drug conditions on 25% of the sessions. Previous studies using these procedures showed a highly significant degree of reliability between the overall scores from observational sessions conducted by pairs of independent observers (Sannerud et al., 1989).

2.4. Drug administration in non-dependent baboons

Acute dose-response functions for the benzodiazepine agonist midazolam, the antagonist flumazenil and two inverse agonists, FG7142 and βCCE were generated in non-dependent baboons. Although both FG7142 and βCCE are often described as inverse agonists, there are a variety of biochemical and pharmacological differences between these compounds (Petersen et al., 1982, 1983). A single dose of test drug was administered immediately before each observational session: midazolam (0.01-3.2 mg/kg i.m.; baboons BE, MI, TH, AR), flumazenil (0.1-32 mg/kg i.m.; baboons BE, MI, TH), FG7142 (0.1-100 mg/kg i.m.; baboons MI, TH, OP) and βCCE (0.1-32 mg/kg, i.m.; baboons BE, MI, TH, AR, OM). Seven to ten days elapsed between injections of individual drug doses.

Interactions among the benzodiazepine agonist midazolam, the antagonist flumazenil, and the inverse agonist βCCE were also studied in non-dependent

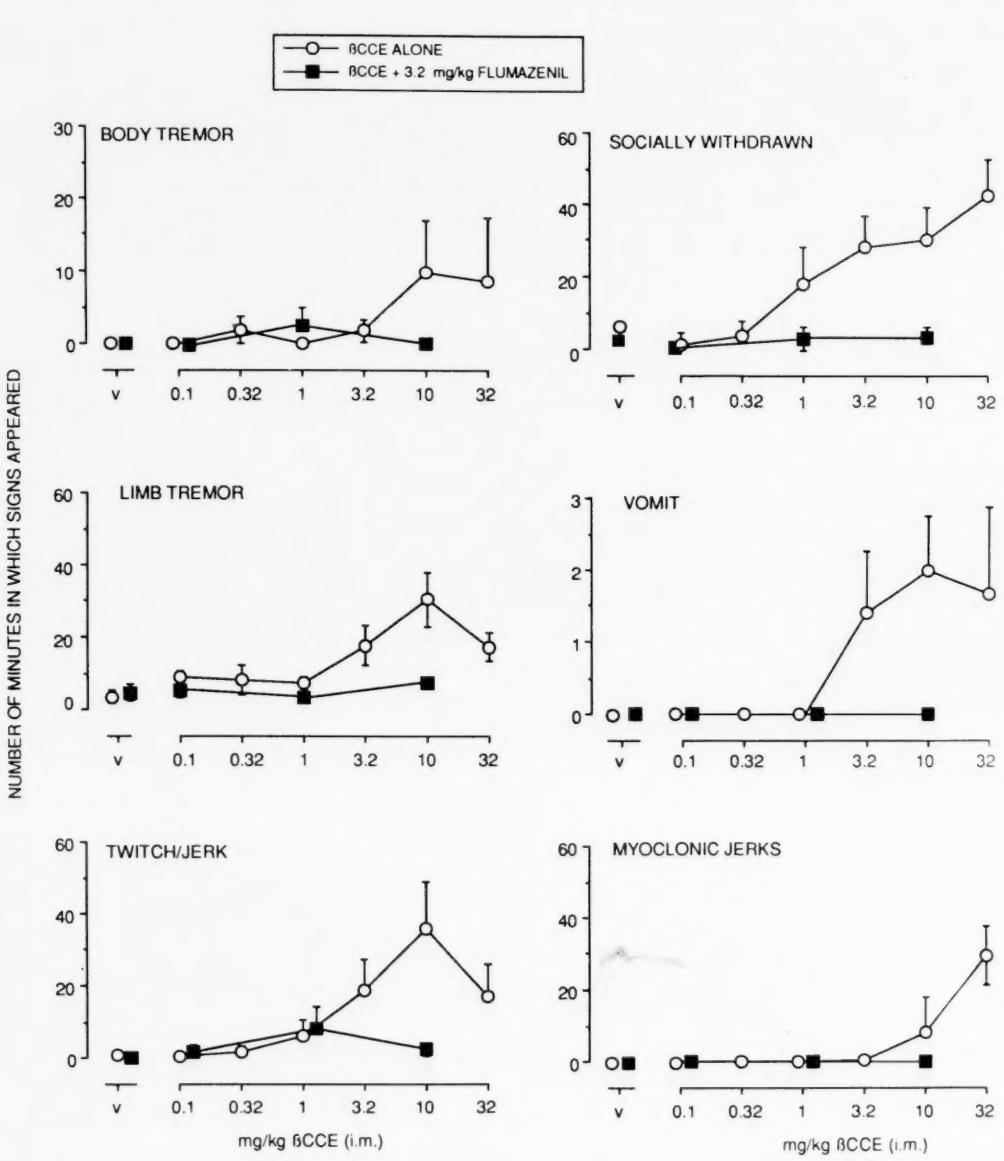

Fig. 3. Behavioral effects of βCCE alone (n = 5) and βCCE in combination with 3.2 mg/kg flumazenil (n = 5) in non-dependent condition. Dose-effect functions for βCCE alone are redrawn from fig. 1. Other details as in fig. 1.

baboons to assess the ability of the benzodiazepine ligands to mutually antagonize their behavioral effects. Three studies were conducted. First, a single dose of flumazenil was administered in combination with a range of βCCE doses: flumazenil (3.2 mg/kg i.m.) and βCCE (0.1, 1.0, 10 mg/kg i.m.) (baboons BE, MI, TH, AR, OM). Second, a single dose of midazolam was administered in combination with a range of βCCE doses: midazolam (0.32 mg/kg i.m.) and βCCE (0.1, 1.0, 10 mg/kg i.m.) (baboons BE, MI, TH, AR). Third, a single dose of midazolam was administered in combination with a single dose of flumazenil: midazolam (3.2 mg/kg i.m.) and flumazenil (3.2 mg/kg i.m.) (baboons TH, AR, OM).

2.5. Drug administration in dependent baboons

Acute dose-response functions for the benzodiazepine agonist midazolam, the antagonist flumazenil and the inverse agonist βCCE were generated in benzodiazepine dependent baboons maintained on 20 mg/kg per day diazepam via continuous i.g. infusion. A single dose of test drug was administered immediately before an observational session: midazolam (0.32-100 mg/kg i.m.; baboons CX, NU), flumazenil (1.0-100 mg/kg i.m.; baboons CH, NU, OM) and βCCE (0.32-100 mg/kg i.m.; baboons CH, NU, OM, AR). Ten to fourteen days elapsed between injections of individual drug doses.

Combinations of the benzodiazepine antagonist flumazenil and the inverse agonist βCCE were also administered to dependent baboons in order to assess the ability of flumazenil to antagonize or potentiate the behavioral effects of βCCE. Two doses of flumazenil were administered in combination with a range of βCCE doses: flumazenil (3.2, 10 mg/kg i.m.) and βCCE (0.32, 3.2, 32 mg/kg i.m.) (baboons CH, NU, OM).

2.6. Drug administration in withdrawn baboons

Acute dose-response functions for flumazenil and βCCE were generated in baboons during diazepam withdrawal. In this experiment, diazepam-dependent baboons were administered vehicle via continuous i.g. infusion for 4 days prior to each dose of flumazenil or βCCE. The baboons were returned to the chronic 20 mg/kg per day i.g. diazepam condition for two weeks after each dose of flumazenil or βCCE. Each dose of flumazenil or βCCE was administered immediately before an observational session: flumazenil (0.032-10 mg/kg i.m.; baboons BE, MI, TH, CH, NU), βCCE (0.032-5.6 mg/kg i.m.; baboons BE, MI, TH, CH). Eighteen days elapsed between injections of individual

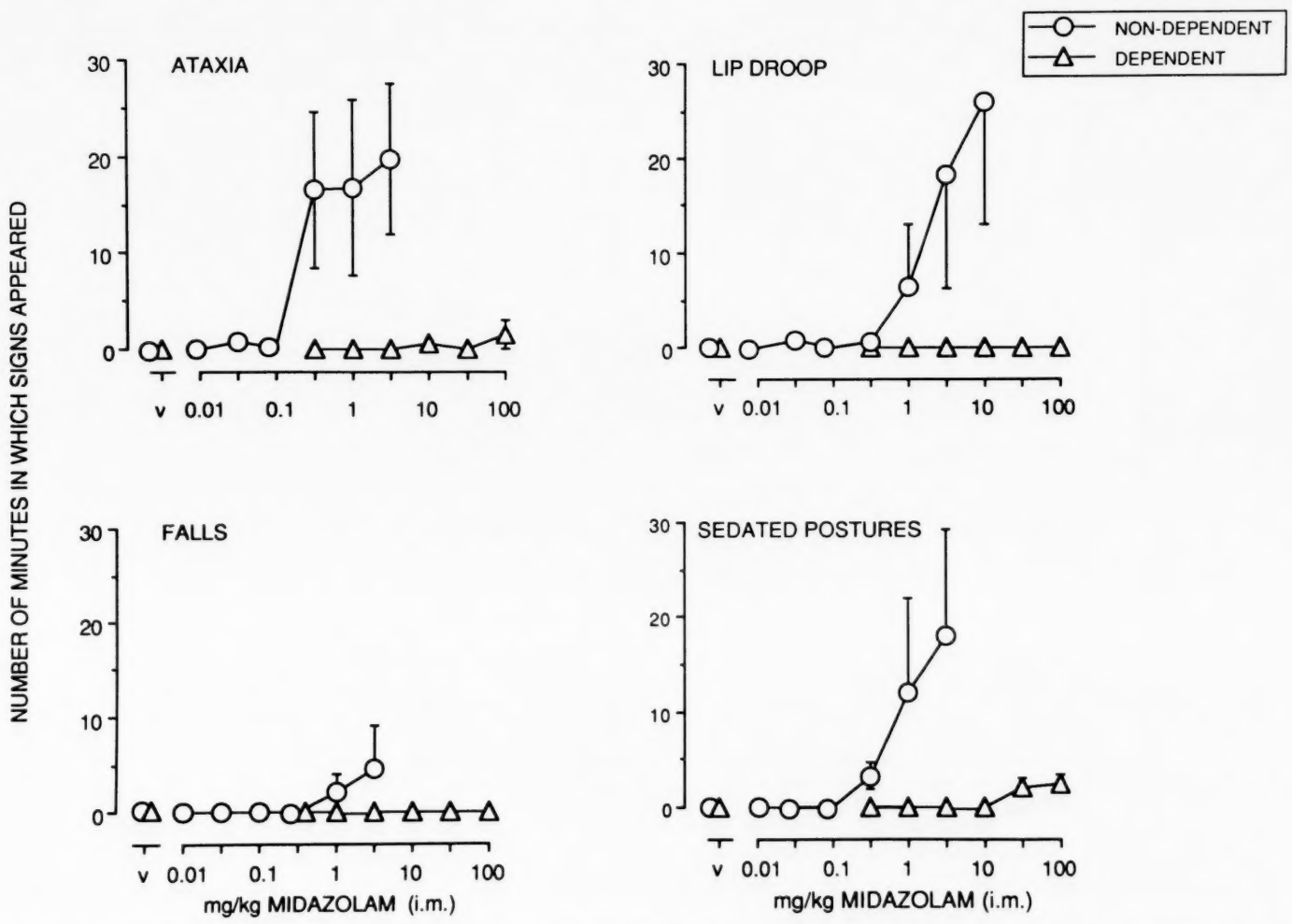

Fig. 4. Behavioral effect of midazolam in the non-condition and diazepam-dependent conditions. Ataxia dose-effect function for midazolam in the non-dependent condition (n = 3) is redrawn from fig. 1. Baboons in the diazepam-dependent condition were maintained on 20 mg/kg per day i.g. diazepam (n = 2). Details as in fig. 1.

drug doses: 14 days of chronic diazepam administration and 4 days of vehicle administration.

2.7. Drugs

Midazolam maleate was dissolved in 0.9% sodium chloride. βCCE was dissolved in 0.2 N HCl and sodium chloride (pH = 5). Flumazenil (Ro 15-1788) was dissolved in propylene glycol : ethanol : sterile water (40 : 10 : 50 v/v) . FG7142 was dissolved in emulphor and saline. Drug and vehicle solutions were freshly prepared immediately before injections and were administered i.m. in volumes of 1.0-3.0 ml. Diazepam for continuous i.g. administration was suspended in 4 g/l suspending agent (BIO-Serv, Frenchtown, NJ). Diazepam, midazolam and flumazenil were provided by Hoffmann-LaRoche (Nutley, NJ). FG7142 was provided by Schering A.G. (Berlin, FRG).

2.8. Data analysis

The occurrence of signs was quantified as the number of minutes in which the sign appeared within the observational session. Data are graphically represented as the group mean ± S.E.

3. Results

3.1. Acute dose-effect functions in non-dependent baboons

The qualitative and quantitative differences in the profile of the behavioral effects produced by the benzodiazepine agonist, antagonist and inverse agonists in non-dependent baboons are shown in fig. 1.

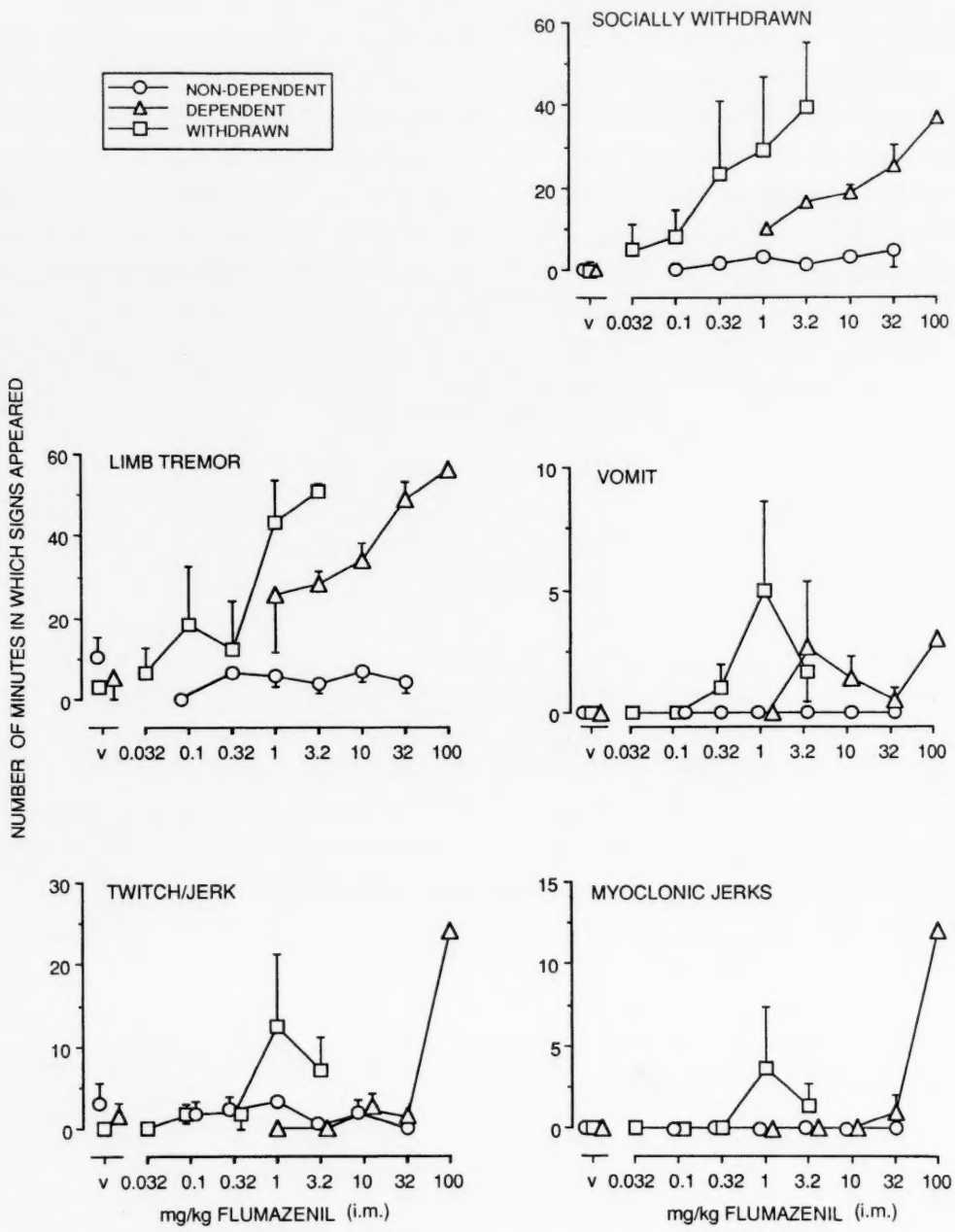

Fig. 5. Behavioral effect of flumazenil in non-dependent, diazepam-dependent and diazepam-withdrawn conditions. Baboons in the diazepam-dependent condition were maintained on 20 mg/kg per day i.g. diazepam (n = 3). Baboons in the diazepam-withdrawn condition were diazepam-dependent (20 mg/kg per day i.g. diazepam) and vehicle was substituted for diazepam for 4 days prior to each dose of flumazenil or vehicle (n = 4). Baboons in the withdrawn condition were returned to 20 mg/kg per day i.g. diazepam for 2 weeks after each dose of flumazenil or vehicle. Dose-effect function for flumazenil in the non-dependent condition (n = 3) is redrawn from fig. 1. Details as in fig. 1.

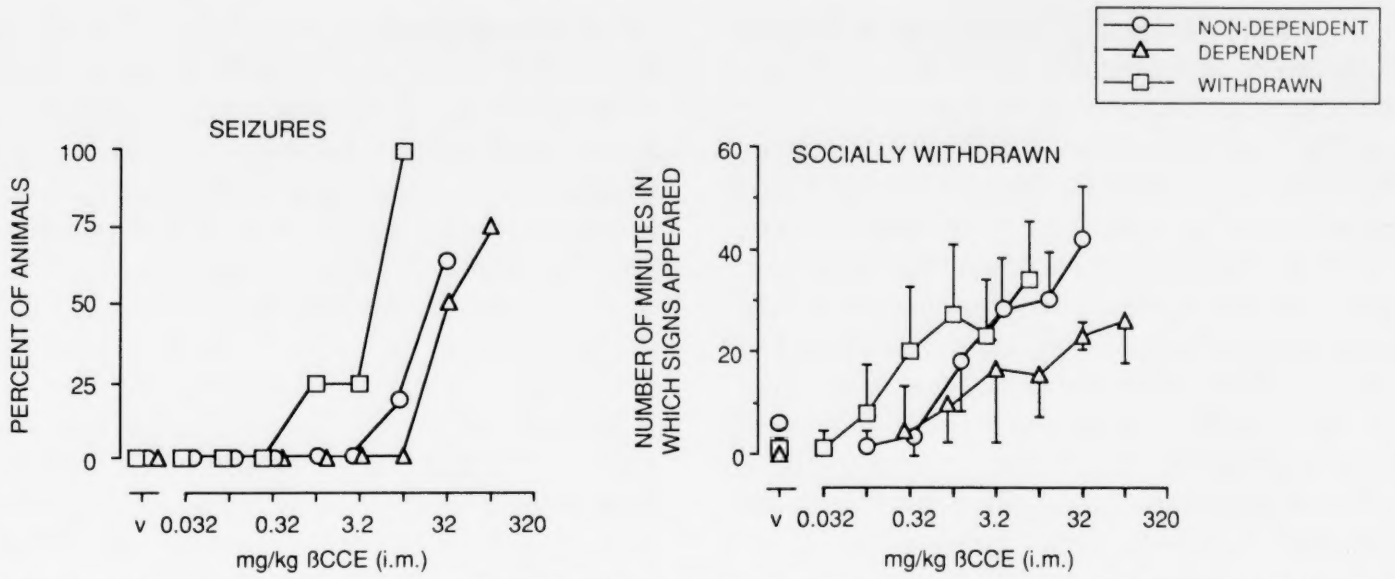

Fig. 6. The behavioral effects of βCCE in the non-dependent (n = 5), diazepam-dependent (n = 4) and withdrawn (n = 4) conditions. X axes: dose of βCCE, log scale. Y axes: percentage of baboons (left panel) or the number of min during the 60-min observational session in which the signs appeared (right panel). Other details as in fig. 5.

Midazolam, the benzodiazepine agonist, produced dose-related increases in signs indicating sedation: ataxia (fig. 1), lip droop, falls and sedated postures (i.e. sitting or laying down with eyes closed) (fig. 4, non-dependent condition). None of these effects were produced by midazolam vehicle or the other drugs tested.

Flumazenil, the benzodiazepine receptor antagonist, was behaviorally inactive: it did not produce a meaningful change in any of the behavioral signs across the wide range of doses evaluated. Figure 1 illustrates the lack of behavioral effect of flumazenil across six classes of signs that were sensitive to one or more of the other drugs tested.

The inverse agonists, FG7142 and βCCE, produced unique profiles of behavioral effects that were different than those observed after midazolam and flumazenil administration. The effects produced by FG7142 and βCCE were similar in that both produced dose-related increases in socially withdrawn posture and limb tremor (fig. 1). The compounds were different in that FG7142 produced dose-related increases in nose rubbing (not shown), while βCCE increased vomiting and several proconvulsant behaviors (twitch/jerks: isolated spasms of head or limbs, and myoclonic jerks: symmetrical clonic spasms of bilateral limbs usually preceding seizures). In addition, βCCE (32 mg/kg) produced seizures (major convulsion or tonic spasms followed by clonic jerking and prolonged depression) in three of the five baboons (fig. 6, non-dependent condition).

3.2. Drug interactions in the non-dependent condition

Assessment of the interactions between midazolam, flumazenil and βCCE showed that each ligand could antagonize the behavioral effects of each of the other ligands.

Figure 2 shows the dose-response functions for βCCE alone and βCCE in combination with a behaviorally active dose of midazolam. As shown, 0.32 mg/kg midazolam produced an increase in ataxia when administered with βCCE vehicle; βCCE produced dose-dependent antagonism of this effect. The figure also shows that the effects produced by 10 mg/kg βCCE alone (socially withdrawn posture, limb tremor, vomiting, twitch/jerks and myoclonic jerks) were completely antagonized by midazolam.

Figure 3 shows the dose-response functions for βCCE alone and βCCE in combination with a dose of flumazenil. Consistent with the results shown in fig. 1, 3.2 mg/kg flumazenil administered with βCCE vehicle did not alter any of the behavioral signs evaluated. Figure 3 shows that the behavioral signs produced by 10 mg/kg βCCE alone (body tremor, socially withdrawn posture, limb tremor, vomiting, twitch/jerk and myoclonic jerks) were completely antagonized by flumazenil.

Not shown graphically, flumazenil (3.2 mg/kg) completely antagonized the the sedative signs produced by midazolam (3.2 mg/kg): ataxia, lip droop, falls and sedated postures (e.g. sitting or laying down with eyes closed).

3.3. Comparison of dose-effect functions in non-dependent, diazepam dependent and withdrawn conditions

There were quantitative and qualitative differences among the dose-effect functions generated for the benzodiazepine ligands in the three conditions of this study.

Figure 4 shows that midazolam was virtually without effect in dependent baboons, in contrast to the dose-

dependent increases in ataxia, lip droop, falls and sedated postures observed in non-dependent baboons.

Figure 5 shows that in contrast to flumazenil's lack of effects in the non-dependent condition, in dependent and withdrawn conditions, flumazenil produced dose-related increases in signs previously shown sensitive to the inverse agonist. Not shown in the fig., in no condition did flumazenil affect signs previously shown sensitive to midazolam (ataxia, lip droop, falls, sedated postures). In addition, flumazenil was substantially more potent in its ability to produce these effects in the withdrawn than in the dependent condition: there were 3.2- to 100-fold differences in the peak effects for socially withdrawn posture, limb tremor, vomiting, twitch/jerks and myoclonic jerks in withdrawn baboons compared to dependent baboons.

βCCE tended to be more potent in producing seizures in the withdrawn condition than in the non-dependent and dependent conditions (fig. 6, left panel). For example, βCCE (10 mg/kg) produced tonic-clonic seizures in all four of the withdrawn baboons, but produced seizures in only one of the five non-dependent baboons, and in none of the four diazepam-dependent baboons. The increased frequency of seizure in the withdrawn condition modified the expression of other behavioral signs during the observational sessions. The βCCE-produced convulsions, which typically occurred 15-20 min after injection, were followed by a prolonged period of post-ictal behavioral depression, which is reflected in the dose-related increases in socially withdrawn postures (fig. 6, right panel).

3.4. Drug interactions in the diazepam-dependent condition

Flumazenil administered in combination with βCCE in the diazepam-dependent condition did not consistently potentiate or antagonize βCCE's behavioral effects. Compared with βCCE alone, flumazenil (3.2 and 10 mg/kg) in combination with βCCE (0.32, 3.2 and 32.0 mg/kg) produced alterations in the frequency of withdrawal signs, such as vomiting, myoclonic jerks, and seizures, but these effects did not appear to be dose-dependent (data not shown).

4. Discussion

The present study found that the behavioral effects of benzodiazepine agonists, antagonists, and inverse agonists can be differentiated using a behavioral rating scale, and that the behavioral effects observed in the non-dependent condition can be modified by chronic diazepam administration in the dependent and withdrawn conditions. In the non-dependent condition, the behavioral effects of benzodiazepine receptor ligands were dose-dependent and appeared to lie on the continuum of behavioral activity ranging from benzodiazepine agonist to benzodiazepine inverse agonist effects: midazolam produced sedation and ataxia, flumazenil produced no behavioral effects, FG7142 produced limb tremor, and βCCE produced twitch/jerks, vomiting, myoclonic jerks and seizures. However, in the diazepam-dependent and withdrawn conditions, the spectrum of behavioral effects produced by benzodiazepine receptor ligands appeared shifted in the direction of the inverse agonist: midazolam produced no effect in the dependent condition, flumazenil produced limb tremor, twitch/jerks, vomiting, and socially withdrawn postures in both dependent and withdrawn conditions, and βCCE was more potent at producing seizures in the withdrawn than in the non-dependent condition.

The present study behaviorally differentiated between FG7142 and βCCE on the basis of their convulsant effects; FG7142 produced only limb tremor, and βCCE produced twitch/jerks, myoclonic jerks and seizures. Although both compounds are often labelled as inverse agonists and have been shown to produce proconvulsant effects under some conditions (Thiébot, et al., 1988), the present results are consistent with preclinical research using an electroshock seizure model in mice that demonstrated that, in contrast to βCCE, FG7142 produces anticonvulsant effects at high doses (Petersen et al., 1983). It is possible that the absence of convulsant effects after FG7142 noted in the present study reflects the lower intrinsic activity at the benzodiazepine receptor complex of FG7142 compared to βCCE.

The production of differential behavioral effects by benzodiazepine receptor ligands in baboons is consistent with the 3-state receptor model of the GABA/benzodiazepine receptor complex; the wide range of behavioral effects produced by benzodiazepine receptor ligands can be related to their bidirectional modulatory effect on the inhibitory neurotransmitter GABA (Polc et al., 1982; Haefely, 1990; Little et al., 1987) . Acute administration of benzodiazepine agonists increase GABA functioning and produce ataxia and sedation in animals and humans (cf. Richards et al., 1986; Haefely, 1988; Woods et al., 1987) . The benzodiazepine antagonist, flumazenil, has virtually no intrinsic effects on GABA in animals or humans at doses that antagonize benzodiazepine agonists (cf. Klotz and Kanto, 1988), although flumazenil has been reported to produce weak agonist or inverse agonist effects when administered in high doses (cf. File and Hitchcott, 1990). The inverse agonists FG7142 and βCCE are reported to decrease GABA functioning, and produce behavioral agitation in animals and 'anxiogenic', effects in humans (cf. Nutt, 1983; Dorow et al., 1987; Thiébot et al., 1988).

In the present study, the behavioral effects produced by the benzodiazepine ligands in the non-dependent condition appeared to be receptor-mediated; administration of combinations of receptor ligands (midazolam + βCCE, midazolam + flumazenil, or flumazenil + βCCE) showed that each ligand could antagonize the effects of the other ligands. In the non-dependent condition, flumazenil antagonized the effects of both midazolam and βCCE, and co-administration of midazolam and βCCE produced a mutual antagonism of their behavioral effects. Consistent with the results in the present study, other investigators found that the interactions of benzodiazepine receptor agonists, antagonists and inverse agonists resulted in antagonism or mutual antagonism of many of their classic behavioral effects, as well as their discriminative stimulus effects (Oakley and Jones, 1980; Tenen and Hirsh, 1980; File and Baldwin, 1987; Ongini et al., 1983; Shannon et al., 1988; Valin et al., 1982; Schweri et al., 1982; File and Pellow, 1984; Dorow et al., 1983; Takada et al., 1986; Barrett et al., 1985; Wettstein 1989).

Relative to the non-dependent condition, chronic diazepam administration altered the behavioral effects of the receptor ligands: (1) midazolam administration produced no behavioral effect in the dependent condition (i.e. tolerance developed to the sedative effects of midazolam which were observed in the non-dependent condition); (2) in contrast to flumazenil's lack of behavioral effects in the non-dependent condition, flumazenil administration in the diazepam-dependent and withdrawn conditions precipitated a withdrawal syndrome that consisted of an inverse agonist-like profile of behavioral effects; (3) βCCE produced seizures in the withdrawn condition at doses that were substantially lower than those required to produce seizures in the non-dependent and dependent conditions; (4) in contrast to flumazenil's antagonism of βCCE's behavioral effects in the non-dependent condition, flumazenil given in combination with βCCE in the dependent condition produced changes in the frequency of withdrawal signs, but did not consistently potentiate or antagonize the effects of βCCE.

These alterations in behavioral effects confirm and extend previous research suggesting that chronic administration of benzodiazepine agonists produce changes in the behavioral effects of the receptor ligands, with the effects shifting along a continuum of activity toward the direction of the inverse agonist (Little et al., 1986; Nutt and Costello, 1988) . This phenomenon, which has been termed 'withdrawal shift', can be observed as a precipitated withdrawal syndrome after administration of an intrinsically neutral receptor antagonist or as potentiation of the behavioral effects of inverse agonists. Flumazenil, for example, had no effect on behavior when given alone, but produced a precipitated withdrawal syndrome during chronic benzodiazepine agonist administration in rats, cats, dogs and baboons (Boisse et al., 1986, 1990; Wilson and Gallager, 1988; Giorgi et al., 1989; Löscher et al., 1989; McNichols et al., 1988; Lukas and Griffiths, 1982, 1984; Lamb and Griffiths 1984; Sannerud et al., 1989). In addition, three benzodiazepine antagonists (CGS 9895, β-CCtB, flumazenil) that had no effect on schedule-controlled behavior, produced disruptions of this behavior during chronic administration of chlordiazepoxide in rats (Takada et al., 1989) . An analogous shift in potency of inverse agonists after termination of flurazepam or lorazepam treatment was observed as sensitization to the convulsant effects in rodent (Little et al., 1987; Nutt and Costello, 1988; Petersen and Jensen 1987a, b; Schatzki et al., 1989). Taken together, these data suggest that chronic administration of benzodiazepine agonists induce an increase in efficacy or potency of the benzodiazepine receptor ligands to produce inverse agonist-like effects.

In the dependent condition of the present study, the insensitivity to βCCE and flumazenil may be explained by a competitive interaction of diazepam with these drugs at the receptor. It is probable that chronic infusion of diazepam produced an accumulation of high concentrations of diazepam and its active metabolites. It seems likely that the presence of those receptor agonists in the dependent condition could have resulted in rightward shifts in the dose-response functions for flumazenil and βCCE compared to the withdrawn condition. These data are consistent with previous reports of a lack of a precipitated withdrawal syndrome by β-carboline derivatives FG 7142 and ZK 93426 in diazepam-dependent cats and the suggestion that this inability may be due to pharmacokinetic factors, such as the presence of high brain levels of diazepam or its metabolites at the receptor (Ongini et al., 1985; Giorgi et al., 1988).

Chronic diazepam administration has been shown to produce a number of biochemical alterations that could possibly influence the expression of the behavioral effects of benzodiazepine receptor ligands. The change in behavioral efficacy of benzodiazepine receptor ligands during chronic diazepam administration may be related to perturbation in the GABA/benzodiazepine-chloride receptor complex, including receptor down-regulation, long-term decreases in GABA sensitivity, changes in GABA/benzodiazepine coupling and/or decreases in GABA efficacy (Gonsalves and Gallager, 1985; Gallager et al., 1985; Heninger and Gallager, 1988; Tietz et al., 1989). In addition, chronic diazepam administration has been reported to increase the production of a putative endogenous inverse agonist-like ligand, diazepam binding inhibitor (DBI) and its precursors (Miyata et al., 1987). Although biochemical changes during chronic diazepam administration are likely to underlie the decreased effectiveness of benzo-

diazepine agonists (i.e. tolerance), and the increased efficacy or potency of flumazenil and βCCE to produce convulsant effects (sensitization) in the dependent and withdrawn conditions, the precise mechanism(s) responsible for these behavioral alterations are unknown and warrant further study.

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References

Barrett, J.E., L.S. Brady, J.M. Witkin, J.M. Cook and P. Larscheid, 1985, Interactions between the benzodiazepine receptor antagonist Ro 15-1788 (flumazenil) and the inverse agonist beta-CCE in behavioral studies with squirrel monkeys, Life Sci. 36, 1407.

Boisse, N.R., R.M. Periana, J.J. Guarino, H.S. Krueger and G.M. Samoriski, 1986, Pharmacological characterization of acute chlordiazepoxide dependence in the rat, J. Pharmacol. Exp. Ther. 239, 775.

Boisse, N.R., N. Quaglietta, G.M. Samoriski and J.J. Guarino, 1990, Tolerance and physical dependence to a short acting benzodiazepine, midazolam, J. Pharmacol. Exp. Ther. 252, 1125.

Braestrup, C., M. Nielsen, T. Honoré, L.H. Jensen and E.N. Petersen, 1983, Benzodiazepine receptor ligands with positive and negative efficacy, Neuropharmacology 22, 1451.

Cumin, R., E.P. Bonetti, R. Scherschlicht and W.E. Haefely, 1982, Use of the specific benzodiazepine antagonist, Ro 15-1788, in studies of physiological dependence on benzodiazepines, Experiencia 38, 833.

Dorow, R., R. Horowsky, G. Paschelke, M. Amin and C. Braestrup, 1983, Severe anxiety induced by FG7142, a β-carboline ligand for benzodiazepine receptors, Lancet 2, 98.

Dorow, R., T. Duka, N. Sauerbrey and L. Höller, 1987, Beta-carbolines: new insights into the clinical pharmacology of benzodiazepine receptor ligands, in: Clinical Pharmacology in Psychiatry, eds. S. Dahl, L.F. Gram, S.M. Paul and W.Z. Potter (Springer-Verlag, Berlin) p. 37.

File, S.E., 1985, Tolerance to the behavioral actions of benzodiazepines, Neurosci. Biobehav. Rev. 9, 113.

File, S.E. and H.A. Baldwin, 1987, Effects of beta-carbolines in animal models of anxiety, Brain Res. Bull. 19, 293.

File, S.E. and P.K. Hitchcott, 1990, A theory of benzodiazepine dependence that can explain whether flumazenil will enhance or reverse the phenomenon, Psychopharmacology, 101, 525.

File, S.E. and S. Pellow, 1984, The anxiogenic effects actions of FG 7142 in the social interaction test is reversed by chlordiazepoxide and Ro 15-1788, but not CGS 8216, Arch. Int. Pharmacodyn. Ther. 271, 198.

Gallager, D.W., A.B. Malcolm, S.A. Anderson and S.F. Gonsalves, 1985, Continuous release of diazepam: Electrophysiological, biochemical and behavioral consequences, Brain Res. 342, 26.

Giorgi, O., M.G. Corda, A. Fernandez and G. Biggio, 1988, The abstinence syndrome in diazepam-dependent cats is precipitated by Ro 15-1788 and Ro 15-4513 but not by the benzodiazepine receptor antagonist ZK 93426, Neurosci. Lett. 88, 206.

Giorgi, O., M.G. Corda, A. Fernandez and G. Biggio, 1989, The beta-carboline derivatives ZK 93426 and FG 7142 fail to precipitate abstinence signs in diazepam-dependent cats, Pharmocol. Biochem. Beh. 32, 671.

Gonsalves, S.F. and D.W. Gallager, 1985, Spontaneous and Ro 15-1788-induced reversal of subsensitivity to GABA following chronic benzodiazepines, Eur. J. Pharmacol. 110, 163.

Gonsalves, S.F. and D.W. Gallager, 1987, Time course for development of anticonvulsant tolerance and GABAergic subsensitivity after chronic diazepam, Brain Res. 405, 94.

Haefely, W., 1988, Endogenous ligands of the benzodiazepine receptor, Pharmacopsychiatry 21, 43.

Haefely, W., 1990, The GABA-benzodiazepine interaction fifteen years later, Neurochem. Res. 15, 169.

Heninger, C. and D.W. Gallager, 1988, Altered gamma-aminobutyric acid/benzodiazepine interaction after chronic diazepam exposure, Neuropharmacology 27, 1073.

Klotz, U. and J. Kanto, 1988, Pharmacokinetics and clinical use of flumazenil (Ro15-1788), Clin. Pharmacokinetics, 14, 1.

Lamb, R.J. and R.R. Griffiths, 1984, Precipitated and spontaneous withdrawal in baboons after chronic dosing with lorazepam and CGS 9896, Drug Alcohol Dependence 14, 11.

Little, H.J., D.J. Nutt and S.C. Taylor, 1986, The effects of drugs acting at the $GABA_A$-receptor/ionophore after chemical kindling with the benzodiazepine receptor ligand FG 7142, Br. J. Pharmacol. 88, 507.

Little, H.J., D.J. Nutt and S.C. Taylor, 1987, Bidirectional effects of chronic treatment with agonists and inverse agonists at the benzodiazepine receptor, Brain Res. Bull. 19, 371.

Löscher, W. and D.N. Stephens, 1988, Chronic treatment with diazepam or the inverse benzodiazepine receptor agonist FG7142 causes differential changes in the effects of GABA stimulation, Epilepsy Res. 2, 253.

Löscher, W., D. Hönack and C.P. Fabbender, 1989, Physical dependence on diazepam in the dog: precipitation of different abstinence syndromes by the benzodiazepine receptor antagonist Ro 15-1788 and ZK 93426, Br. J. Pharmacol. 97, 843.

Lukas, S.E. and R.R. Griffiths, 1982, Precipitated withdrawal by a benzodiazepine receptor antagonist (Ro 15-1788) after 7 days of diazepam, Science 217, 1161.

Lukas, S.E. and R.R. Griffiths, 1984, Precipitated diazepam withdrawal in baboons: Effects of dose and duration of diazepam exposure, Eur. J. Pharmacol. 100, 163.

Lukas, S.E., R.R. Griffiths, L.D. Bradford, J.V. Brady and L. Daley, 1982, A tethering system for intravenous and intragastric drug administration in the baboon, Pharmacol. Biochem. Behav. 17, 823.

McNichols, L., W.R. Martin, J. Sloan and E. Wala, 1988, Precipitation of abstinence in nordiazepam and diazepam dependent dogs, J. Pharmacol. Exp. Ther. 245, 221.

Miyata, M., I. Mocchetti, C. Ferrarese, A. Guidotti, E. Costa, 1987, Protracted treatment with diazepam increases turnover of putative endogenous ligands for the benzodiazepine/β-carboline recognition site, Proc. Natl. Acad. Sci. 84, 1444.

Nutt, D., 1983, Pharmacological and behavioural studies of benzodiazepine antagonists and contragonists, in: Benzodiazepine Recognition Site Ligands: Biochemistry and Pharmacology, eds. G. Biggio and E. Costa (Raven Press, New York) p. 153.

Nutt, D.J. and M.J. Costello, 1988, Rapid induction of lorazepam dependence and reversal with flumazenil, Life Sci. 43, 1045.

Oakley, N.R. and B.J. Jones, 1980, The proconvulsant and diazepam reversing effects of ethyl β-carboline-3-carboxylate, Eur. J. Pharmacol. 68, 381.

Ongini, E., C. Barzaghi and M. Marzanatti, 1983, Intrinsic and antagonistic effects of β-carboline FG 7142 on behavioral and EEG actions of benzodiazepines and pentobarbital in cats, Eur. J. Pharmacol. 95, 125.

Ongini, E., M. Marzanatti, F. Bamonte, A. Monopoli and V. Guzzon, 1985, A beta-carboline antagonizes benzodiazepine actions

but does not precipitate the abstinence syndrome in cats, Psychopharmacology 86, 132.

Petersen, E.N. and L.H. Jensen, 1987a, Chronic treatment with lorazepam and FG 7142 may change the effects of benzodiazepine receptor agonists, antagonists and inverse agonists by different mechanisms, Eur. J. Pharmacol. 133, 309.

Petersen, E.N. and L.H. Jensen, 1987b, Lorazepam and FG 7142 induce tolerance to the DMCM antagonistic effect of benzodiazepine receptor ligands, Brain Res. Bull. 19, 187.

Petersen, E.N., G. Paschelke, W. Kehr, M. Nielsen and C. Braestrup, 1982, Does the reversal of the anticonflict effect of phenobarbital by βCCE and FG7142 indicate benzodiazepine receptor-mediated anxiogenic properties? Eur. J. Pharmacol. 82, 217.

Petersen, E.N., L.H. Jensen, T. Honoré and C. Braestrup, 1983, Differential pharmacological effects of benzodiazepine receptor inverse agonists, in: Benzodiazepine Recognition Site Ligands: Biochemistry and Pharmacology, eds. G. Biggio and E. Costa (Raven Press, New York) p. 57.

Polc, P., P.E. Bonetti, R. Schaffner and W. Haefely, 1982, A three-state model of the benzodiazepine receptor explains the interactions between the benzodiazepine tranquilizers, β-carbolines and phenobarbitone, Naunyn-Schmiedeb. Arch. Pharmacol. 321, 260.

Richards, J.G., P. Schoch, H. Möhler and W. Haefely, 1986, Benzodiazepine receptors resolved, Experientia 42, 121.

Sannerud, C.A., J.M. Cook and R.R. Griffiths, 1989, Behavioral differentiation of benzodiazepine ligands after repeated administration in baboons, European J. Pharmacol. 167, 333.

Schatzki, A., F. Lopez, D.J. Greenblatt, R.I. Shader and L.G. Miller, 1989, Lorazepam discontinuation promotes 'inverse agonist' effects of benzodiazepines, Br. J. Pharmacol. 98, 451.

Schweri, M., M. Cain, J. Cook, S. Paul and P. Skolnick, 1982, Blockade of 3-carbomethoxy-β-carboline induced seizures by diazepam and the benzodiazepine antagonists, Ro 15-1788 and CGS 8216, Pharmacol. Biochem. Behav. 17, 457.

Shannon, H.E., T.J. Hagen, F. Guzman and J.A. Cook, 1988, Beta-carbolines as antagonists of the discriminative stimulus effects of diazepam in rats, J. Pharmocol. Exp. Ther. 246, 275.

Takada, K., G. Winger, J. Cook, P. Larscheid and J.H. Woods, 1986, Discriminative and aversive properties of beta-carboline-3-carboxylic acid ethyl ester, a benzodiazepine receptor inverse agonist, in rhesus monkeys, Life Sci. 38, 1049.

Takada, K., T. Suzuki, T. Hagen, J.M. Cook and J.L. Katz, 1989, Behavioral effects of benzodiazepine antagonists in chlordiazepoxide tolerant and non-tolerant rats, Life Sci. 44, 289.

Tenen, S.S. and J.D. Hirsh, 1980, β-Carboline-3-carboxylic acid ethyl ester antagonizes diazepam activity, Nature 288, 609.

Thiébot, M., P. Soubrie and D. Sanger, 1988, Anxiogenic properties of beta-CCE and FG 7142: A review of promises and pitfalls, Psychopharmacology 94, 452.

Tietz, E.I. and H.C. Rosenberg, 1988, Behavioral measurement of benzodiazepine tolerance and GABAergic subsensitivity in the substantia nigra pars reticulata, Brain Res. 438, 41.

Tietz, E.I., T.H. Chiu and N.C. Rosenberg, 1989, Regional GABA/benzodiazepine receptor/chloride channel coupling after acute and chronic benzodiazepine treatment, Eur. J. Pharmacol. 167, 57.

Valin, A., R.H. Dodd, D.R. Liston, P. Potter and J. Rossier, 1982, Methyl-β-carboline-induced convulsions are antagonized by Ro15-1788 and by propyl-β-carboline, Eur. J. Pharmacol. 85, 93.

Wettstein, J.G., 1989, Behavioral studies with the beta-carboline FG 7142 combined with related drugs in monkeys, Eur. J. Pharmacol. 163, 219.

Wilson, M.A. and D.W. Gallager, 1988, Ro15-1788-induced seizures in rats continually exposed to diazepam for prolonged periods, Epilepsy Res. 2, 14.

Woods, J.H., J.L. Katz and G. Winger, 1987, Abuse liability of benzodiazepines, Pharmacol. Rev. 39, 251.

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The effect of vigabatrin on central nervous system oxygen toxicity in rats

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The toxicity of hyperbaric oxygen in the central nervous system is expressed by clinical and electroencephalographic (EEG) manifestations resembling those of generalized tonic-clonic seizures. In the search for drugs effective against these seizures, we tested vigabatrin, an irreversible inhibitor of GABA (γ-aminobutyric acid) transaminase. Five different doses of vigabatrin (ranging from 50 to 500 mg/kg) or vehicle were injected i.p. in rats implanted with cortical electrodes, 4 h prior to exposure to 5 ATA (0.5 MPa) oxygen. EEG and spectral analysis of the background EEG activity were monitored for the different dosages of the drug. The duration of the latent period before the appearance of electrical discharges in the EEG was used as an index of oxygen toxicity. The protective effect of vigabatrin was dose-related, and complete protection against hyperoxic-induced discharges was at 180 mg/kg. The protective effect lasted 24 h and decreased gradually disappearing completely on the third day. An increase in the low frequency bands of the EEG and a decrease in the faster activity were correlated with the vigabatrin dosage injected. Our results suggest that vigabatrin has the potential of being a useful drug in the treatment and prevention of oxygen-induced seizures during hyperbaric oxygen therapy.

Oxygen toxicity; Generalized seizures; Vigabatrin; Hyperoxia; EEG analysis; γ-Vinyl GABA

1. Introduction

Breathing oxygen at high pressure causes dramatic convulsive manifestations known as central nervous system (CNS) oxygen toxicity ('Paul Bert effect') within a time lag inversely proportional to the oxygen partial pressure (Bert, 1943; Bean, 1945; Lambertsen, 1965). These convulsions have been defined and classified as generalized, tonic-clonic (grand mal) seizures (Lambertsen, 1965; Sonnenschein and Stein, 1953; Donald, 1947; Wood, 1972) on the basis of clinical and electroencephalographic (EEG) observations. A typical electroencephalogram consisting of spike and wave discharges is recorded at both hemispheres, starting simultaneously or at random in cortical and subcortical areas (Rucci et al., 1967; Harel et al., 1969). These discharges are believed to be reversible, causing no residual neurological damage, and disappear on reduction of the oxygen pressure.

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** Professor Dan Harel passed away in the winter of 1990. We dedicate this article to the memory of a distinguished physician, researcher and teacher, a unique human being.

Oxygen free radicals, produced in excess while oxygen at high partial pressures is breathed and overwhelming the body's normal antioxidant defence system, seem to mediate the hyperoxic insult (Gerschman et al., 1954; Jamieson, 1989). However, the prime site of their attack, whose end result is the discharges in the whole brain, remains a subject of debate.

γ-Aminobutyric acid (GABA) was suggested as playing a major role in the etiology of hyperoxic-induced seizures, either through its role in oxidative mechanisms (Chance et al., 1965) or by acting as a modulator of nerve transmission (Wood, 1972). The following are the main evidence supporting this suggestion: a specific reversible decrease in the concentration of GABA in the brain prior to the hyperoxic-induced seizures, a correlation between susceptibility to hyperbaric oxygen seizures and rate of decrease in cerebral GABA levels for various species and different ages, and the sensitivity of the GABAergic enzymes to high oxygen pressures, compared to other neurotransmitter enzymes (Wood, 1975). However, in contrast, it is also suggested that hyperoxic seizures, as well as the protection against these seizures, are unrelated to the changes in GABA levels (Alderman et al., 1974).

Hyperbaric oxygen, commonly used in military and professional diving, is an accepted treatment for decompression sickness, carbon monoxide intoxication,

gas gangrene, air embolism, soft tissue infections, traumatic peripheral ischemia, etc. (Mader, 1989). The development of hyperoxic seizures might pose a limitation to exploring the full advantages of hyperbaric oxygen therapy.

On the basis of the evidence in support of GABA's role in the etiology of hyperoxic-induced seizures, we searched for an antiepileptic drug which would cause an increase in brain GABA levels, with minimal side-effects.

Vigabatrin (γ-vinyl GABA), given by a peripheral route, crosses the blood-brain barrier and induces long-term, dose-dependent, irreversible inhibition of brain GABA transaminase (GABA-T), accompanied by a sustained increase in GABA concentration (Jung et al., 1977). Clinical trials have revealed promising results for this compound as a long-lasting antiepileptic drug, with only mild and transient side-effects (Gram et al., 1985).

We tested the efficacy of vigabatrin in the suppression of the hyperbaric oxygen-induced discharges, using as a model a freely moving rat with continuous electroencephalogram monitoring. Apart from the practical application to hyperbaric oxygen therapy, an investigation of the beneficial effect of vigabatrin on hyperoxic-induced discharges might also shed some light on certain features of CNS oxygen toxicity.

2. Materials and methods

Experiments were carried out on male Sprague–Dawley rats weighing 220-280 g, fed on commercial food ad libitum and kept under a normal day/night cycle. Under Equi-Thensin anesthesia, 0.2-0.3 ml/100 g body weight, i.p. (chloral hydrate 1% w/v, nembutal 4% w/v, and $MgSO_4$ 1.3% w/v dissolved in absolute alcohol, propylene glycol, and water), the animal's skull was exposed and five stainless steel screws were inserted into the bone bilaterally over the parietal and posterior cortex. Soldered wires linked the screws to a miniature connector fixed to the bone with self-cure acrylic. After a recovery period of 4 days, a cable with a matching male plug was inserted into the connector so that a continuous two-channel bipolar recording of the electroencephalogram could be obtained from the freely moving rat. The EEG was recorded on an FM tape, Teak XR-50, for further analysis. Each rat was exposed once only to the high oxygen pressure in a 3-l plexiglass cage which was placed inside a 150-l animal hyperbaric chamber (T.C.A.M.O., Roberto Galleazi, Italy) compressed with air. A constant oxygen flow of 2 l/min was used to prevent accumulation of carbon dioxide in the plexiglass cage. Chamber temperature was maintained at 22 °C, with a transient rise in temperature of up to 2 °C during and shortly after compression.

Forty-two rats were assigned independently to six different treatments in a completely randomized design. Each rat was exposed only once to 5 ATA oxygen, after i.p. injection of vigabatrin (Merrell Dow Res. Inst.) in the range of 50-500 mg/kg, or vehicle (saline) in a volume of approximately 0.5 ml.

The rats were observed for behavioral changes such as drowsiness, apathy, ataxia and unsteadiness. Spectral analysis of the EEG was carried out by Fast Fourier Transformation (FFT) on a micro PDP 11-23 computer. The rate of sampling was 124/s, analyzing 16-s data epochs, with a frequency resolution of 1.0 Hz. Before the FFT analysis was carried out, the raw signal in the time domain was multiplied by a Hanning window to reduce the edge effects on the frequency contents. The transformed signal in the frequency domain was smoothed to reduce the error in the frequency computation. Slow DC shifts were eliminated digitally, by filtering below 1 Hz. The relative intensity for each frequency was averaged, then divided into four frequency bands: delta (1-3 Hz), theta (3-7 Hz), alpha (7-13 Hz), and beta (13-25 Hz). Baseline recording was performed at atmospheric pressure in the open hyperbaric chamber, followed by a recording taken 4 h after vigabatrin injection and continuing from compression to 5 ATA (0.5 MPa) until the electric seizures appeared. The chamber was compressed with oxygen up to 5 ATA at the rate of 1 ATA/min. The duration of the latent period from the time the desired pressure was reached until the appearance of the discharges was used as the criterion for CNS oxygen toxicity. Exposure to 5 ATA was terminated and the chamber was immediately decompressed at the rate of 1 ATA/min, following the appearance of the first electrical discharges in the EEG. The experiment was terminated, even if no discharges had been recorded after 50 min, to avoid any complications due to pulmonary oxygen toxicity.

To test the long-term antiepileptic effects of the drug, the rats were exposed to 5 ATA oxygen at 24-h intervals after a single injection of 250 mg/kg vigabatrin (24, 48 and 72 h, six rats for each time interval).

Latent period data are presented as a notched box plot display (Williamson et al., 1989), inserted into the format of a dose-response curve. The box plot displays graphically the 25 and 75 percentiles, the median, and the furthest observations in the group which are still no more than two box-lengths away from the median. Observations outside this range are plotted individually. The notches (indicated here by a shaded area) add information by presenting confidence intervals around the medians. Tukey's test was performed for comparing the non-treated (vehicle) and the vigabatrin-treated groups. Changes in background EEG activity were analyzed by the paired t-test.

3. Results

Figure 1 represents the typical EEG of a rat given a low dose of vigabatrin. Control recordings made at atmospheric pressure and then 4 h after the injection of 50 mg/kg vigabatrin are shown, as is the appearance of the electrical discharges while hyperbaric oxygen at 5 ATA was breathed. No notable change was observed in the background EEG of the vigabatrin-treated rats. These rats exhibited the usual typical form of the hyperoxic-induced discharges.

Higher doses of vigabatrin eliminated the appearance of the discharges, although some slight changes in background EEG activity were occasionally observed: slowing of electrical activity and an increase in amplitude.

The dose-response curve (vigabatrin concentrations vs. duration of latent period for electrical discharges) on exposure to 5 ATA oxygen, 4 h post injection of vigabatrin, is plotted in fig. 2. The duration of the latent period increased gradually, reaching full protection after the injection of 180 mg/kg (all seven rats in the group were without electrical discharges for 50 min). The maximal protective effect was maintained on further increases in the drug dose. Rats injected with 500 mg/kg vigabatrin showed signs of unsteadiness, reflected by unsteady gait, and dulling of senses, reflected by diminished reaction to environmental auditory stimuli.

Figure 3 presents the long-term effect of vigabatrin after a single injection of 250 mg/kg. The full protection (50 min at 5 ATA oxygen) observed 4 h after vigabatrin injection decreased gradually, as expressed in the shortening of the latent period, which had almost reached control values after 72 h.

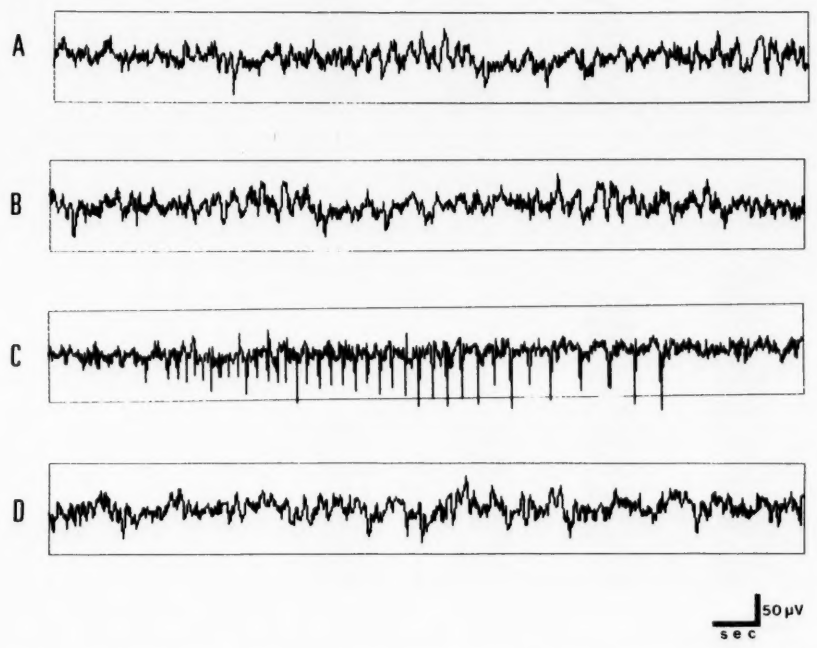

Fig. 1. Typical EEG from a rat treated with 50 mg/kg vigabatrin. (A) Control, air at atmospheric pressure. (B) Four hours post injection. (C) The appearance of the electrical discharges at 5 ATA oxygen. (D) On return to atmospheric pressure. Calibration: 1 s/50 μV.

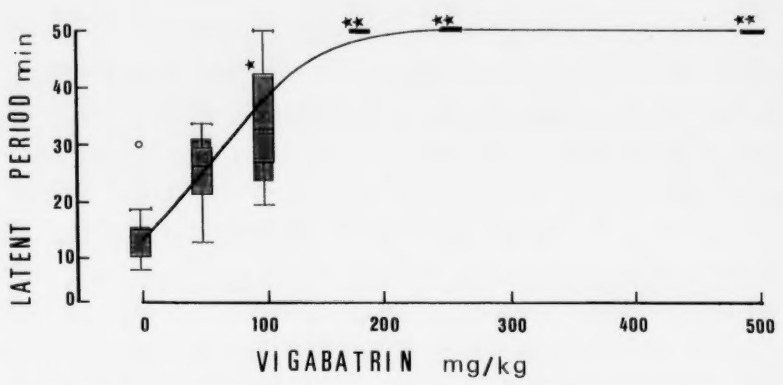

Fig. 2. Dose-response curve: the relationship between the dose of vigabatrin injected and the duration of the latent period preceding the hyperoxic electrical discharges (min). Each concentration is represented by a box plot showing the median, extremes and interquartile range (25-75%) (n = 7 for each group). Observations more than two box-lengths away from the median are plotted individually (a circle). * $P < 0.05$; ** $P < 0.001$, for significance in Tukey's test, compared to vehicle data. Shaded area represents confidence intervals around the medians.

The background EEG activity under various vigabatrin concentrations is shown in fig. 4, demonstrating a significant dose-related increase in the low frequency bands, and a decrease in the faster activity as the dosage of injected vigabatrin was increased (at 500 mg/kg vigabatrin).

4. Discussion

Vigabatrin suppressed hyperbaric oxygen electrical discharges at doses with almost no observed side-effects. The protective effect followed a typical dose-response relationship. Vigabatrin 180 mg/kg provided full protection for as long as 50 min (this time limit ensured safe exposure to 5 ATA oxygen, without the

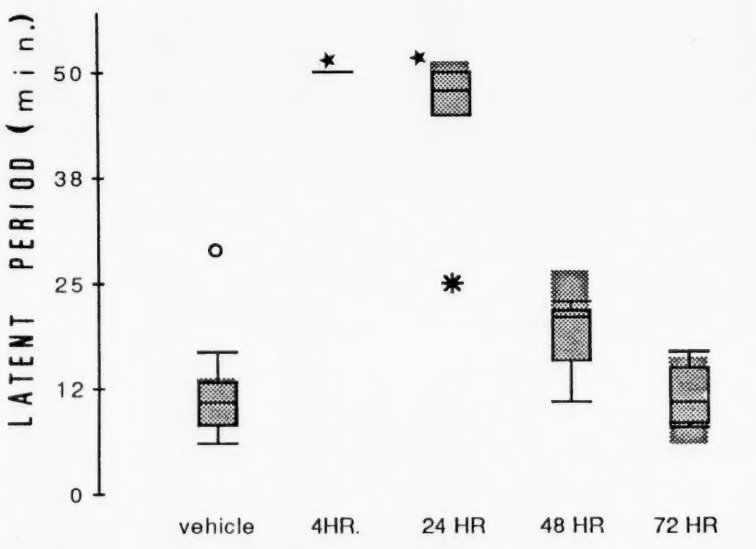

Fig. 3. Time (duration of latent period) until the appearance of electrical discharges in the EEG, on exposure to 5 ATA oxygen at different time intervals after injection of a single dose of 250 mg/kg vigabatrin or vehicle (n = 6 for each group). * Indicates a significance of $P < 0.05$ in Tukey's test, compared to vehicle data. The data are presented as a box plot. Shaded area represents confidence intervals around the medians.

development of pulmonary oxygen toxicity). With a further increase in the vigabatrin dosage, the protection for 50 min was maintained, but side-effects such as unsteadiness and drowsiness were observed. The protective effect of a single dose of the drug lasted for 48 h, suggesting a major advantage of using this drug in patients undergoing a series of hyperbaric oxygen treatments. This prolonged protective effect correlated well with the results of biochemical studies showing a decrease in GABA-T activity and elevation of GABA concentrations in the brain after a single dose of vigabatrin. It lasted for 24 h and returned to normal values after 5 days (Jung et al., 1977; Gale, 1989).

GABA transmission plays a key role in controlling seizure activity. The pharmacological approach to enhancing GABAergic inhibition by the inhibition of GABA transaminase seems to be promising. Two of the major drugs available which act on the principal catabolic enzyme of GABA are valproate and vigabatrin. However, valproate, which has been studied previously in our laboratory, fails to protect against hyperoxic seizures (Harel et al., 1978), while vigabatrin suppresses the seizures. These contradictory results do not deny the importance of GABA in controlling hyperoxic seizures. Although both drugs elevate the level of GABA in the brain, they differ in their specific effect on transmitter-related and non-transmitter-related (glial) GABA pools (Gram, 1988; Gram et al., 1989). In addition, they may act differently upon specific regions of the brain and on pathways which are critical for the control of hyperoxic seizure activity (Gale, 1989; Löscher, 1981; Iadarola et al., 1979). Recently, mechanisms other than GABAergic ones have been suggested to explain the antiepileptic effect of valproate.

Our study suggests the importance of GABAergic mechanisms in controlling discharges induced by hyperbaric oxygen. There could be two points at which vigabatrin exerts its protective effect. At one point, hyperbaric oxygen exerts its toxic effects on specific areas and mechanisms involving GABA, as has been suggested by Wood and others (Bean, 1945; Lambertsen, 1965; Wood, 1975), and vigabatrin antagonizes the deleterious effect of hyperbaric oxygen by increasing GABA levels in the brain. At the other point, there may be involvement of an unspecific mechanism. Even though the toxic effect of oxygen is not to be found within the GABA system, vigabatrin increases the inhibitory tone, or rather controls the gain level of seizure propagating pathways, thus bringing about protection against oxygen toxicity.

Below the dose of 500 mg/kg, at which slight unsteadiness and drowsiness were observed, vigabatrin did not cause any behavioral changes, but the EEG background activity changed at the same time as pro-

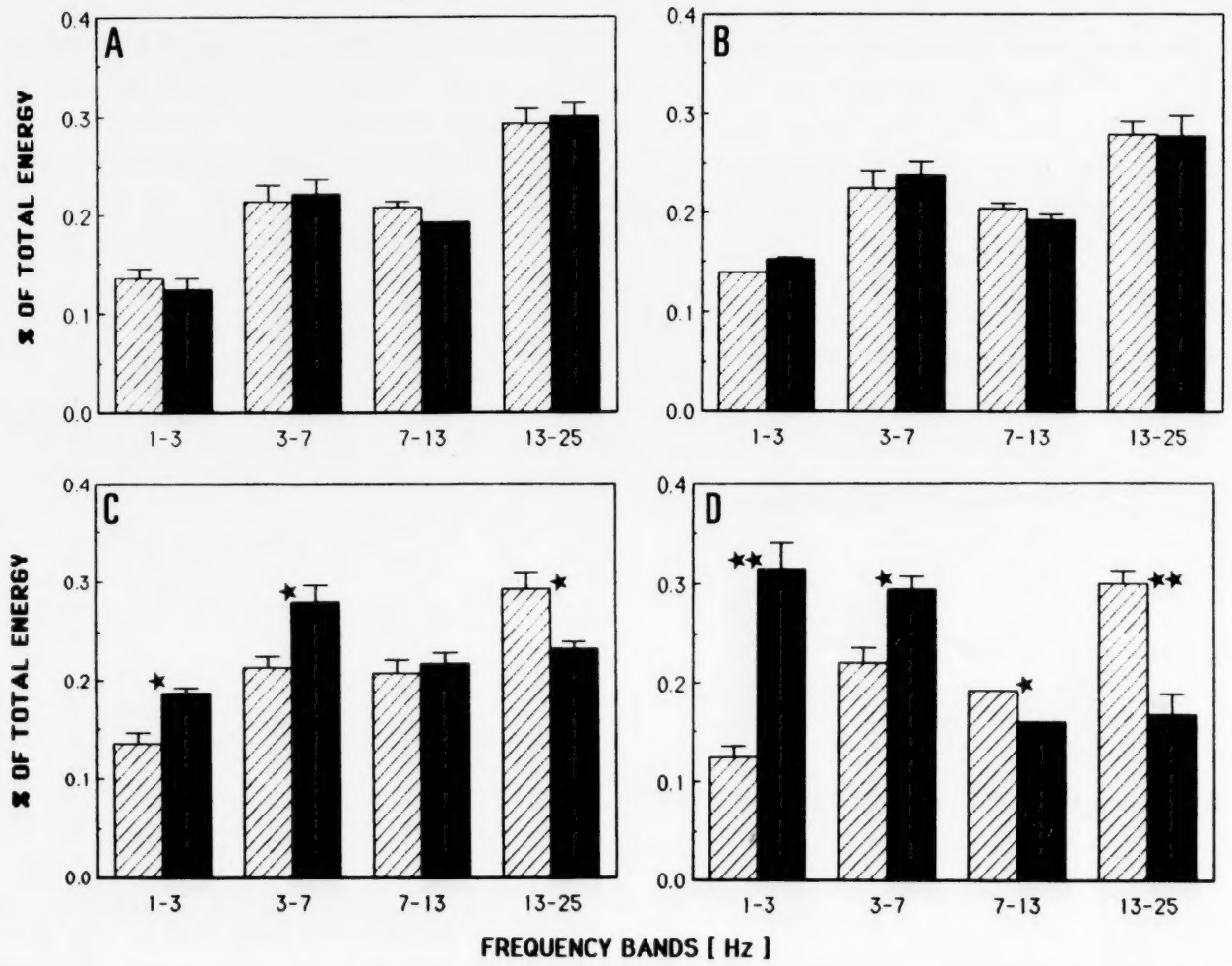

Fig. 4. Relative power (percent of total EEG energy) in four frequency bands after treatment with (A) vehicle (saline); (B) 50 mg/kg vigabatrin; (C) 250 mg/kg vigabatrin and (D) 500 mg/kg vigabatrin. The data are presented as means ± S.E. Hatched bars represent control groups. Full bars represent vigabatrin-injected groups. * Indicates a significance of $P < 0.05$, and ** $P < 0.001$, in a paired t-test.

tection against hyperoxic electrical discharges. Slowing of the EEG, expressed by a significant increase in the delta and theta band and by a decrease in the beta activity (fig. 4C; 250 mg/kg vigabatrin), expanded (fig. 4D: 500 mg/kg) as reflected by the level of significance. At that concentration, a significant decrease was also noted in the relative power of the alpha activity. We do not know of what relevance these background EEG changes are for the function of the brain and the state of the afferent sensory pathways. Only a few changes in evoked responses have been reported to occur under vigabatrin treatment (Cosi et al., 1988) even after long-term use. Vigabatrin seems to be a promising, highly effective, long-lasting antiepileptic compound, which suppresses CNS oxygen toxicity with almost no side-effects. This drug would be of benefit in the pretreatment of epilepsy-prone patients undergoing hyperbaric oxygen therapy, as well as for those who unexpectedly develop symptoms of CNS oxygen toxicity in the course of a series of treatments.

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References

Alderman, J.L., B.W. Culver and M.K. Shellenberger, 1974, An examination of the role of gamma aminobutyric acid (GABA) in hyperbaric oxygen-induced convulsions in the rat. I. Effects of increased gamma aminobutyric acid and protective agents, J. Pharmacol. Exp. Ther. 190, 334.

Bean, J.W., 1945, Effects of oxygen at increased pressure, Physiol. Rev. 25, 1.

Bert, P., 1943, Barometric Pressure. Researches in Experimental Physiology, transl. M.A. Hitchcock and F.A. Hitchcock (College Book Co., Columbus, OH).

Chance, B., D. Jamieson and H. Coles, 1965, Energy linked pyridine nucleotide reduction: inhibitory effect of hyperbaric oxygen in vitro and in vivo, Nature 206, 257.

Cosi, V., R. Callieco, C.A. Galimberti, R. Manni, A. Tartara, G. Lanzi, U. Balottin and E. Perucca, 1988, Effect of vigabatrin (gamma vinyl GABA) on visual, brainstem auditory and somatosensory evoked potentials in epileptic patients, Eur. Neurol. 28, 42.

Donald, K.W., 1947, Oxygen poisoning in man, Br. Med. J. (i), 667.

Gale, K., 1989, GABA in epilepsy: the pharmacologic basis, Epilepsia 30 (Suppl. 3). S1.

Gerschman, R., D.L. Gilbert, S.W. Nye, P. Dwyer and W.O. Fenn, 1954, Oxygen poisoning and x-irradiation: a mechanism in common, Science 119, 623.

Gram, L., 1988, Experimental studies and controlled clinical testing of valproate and vigabatrin, Acta Neurol. Scand. 78, 241.

Gram, L., P. Klosterskov and M. Dam, 1985, Gamma vinyl GABA: A double blind placebo-controlled trial in partial epilepsy, Ann. Neurol. 17, 262.

Gram, L., O.M. Larsson, A. Johnson and A. Schousboe, 1989, Experimental studies of the influence of vigabatrin on the GABA system, Br. J. Clin. Pharmacol. 27 (Suppl. 1), 13S.

Harel, D., D. Kerem and S. Lavy, 1969, The influence of high oxygen pressure on the electrical activity of the brain, Electroencephalogr. Clin. Neurophysiol. 26, 310.

Harel, D., N. Raday-Bitterman and D. Kerem, 1978, Influence of a GABA transaminase inhibitor on central nervous system oxygen toxicity, Aviat. Space Environ. Med. 49, 877.

Iadarola, M.J., A. Raines and K. Gale., 1979, Differential effects of n-dipropylacetate and amino-oxyacetic acid on gamma-aminobutyric acid levels in discrete of rat brain, J. Neurochem. 33, 1119.

Jamieson, D., 1989, Oxygen toxicity and reactive oxygen metabolites in mammals, Free Rad. Biol. Med. 7, 87.

Jung, M.J., B. Lippert, B.W. Metcalf, P. Bohlen and P.J. Schechter, 1977, Gamma-vinyl GABA, a new selective irreversible inhibitor of GABA-T; effects on brain GABA metabolism in mice, J. Neurochem. 29, 797.

Lambertsen, C.J., 1965, Effects of oxygen at high partial pressure, in: Handbook of Physiology, Respiration II, eds. W.D. Fenn and H. Rahn (American Physiological Society, Washington, DC) p. 1027.

Löscher, W., 1981, Valproate induced changes in GABA metabolism at the subcellular level, Biochem. Pharmacol. 30, 1364.

Mader, J.T., 1989, Hyperbaric Oxygen Therapy: A Committee Report (Undersea and Hyperbaric Medical Society, Bethesda, MD).

Rucci, F.S., M.L. Giretti and M. La Rocca, 1967, Changes in electrical activity of the cerebral cortex and of some subcortical centers in hyperbaric oxygen, Electroencephalogr. Clin. Neurophysiol. 22, 231.

Sonnenschein, R.R. and S.N. Stein, 1953, Electrical activity of the brain in acute oxygen poisoning, EEG Clin. Neurophysiol. 5, 521.

Williamson, D.F., R.A. Parker and J.S. Kendrick, 1989, The box plot: a simple visual method to interpret data, Ann. Intern. Med. 110, 916.

Wood, J.D., 1972, Systemic oxygen derangements, in: Experimental Models of Epilepsy, eds. D.P. Purpura, J.K. Penry, D.B. Tower, D.M. Woodbury and R.D. Walter (Raven Press, New York) p. 459.

Wood, J.D., 1975, The role of gamma aminobutyric acid in the mechanism of seizures, Prog. Neurobiol. 5, 79.

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Prevention of neuroleptic-induced dopamine D_2 receptor supersensitivity by chronic iron salt treatment

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The ability of neuroleptics to induce dopamine D_2 receptor supersensitivity has been linked to the onset of tardive dyskinesia, the major side-effect of these drugs. Brain iron metabolism has been shown to be involved in the regulation of dopamine D_2 receptors. We now examined the effect of chronic treatment with $FeCl_2$ on chlorpromazine-induced D_2 receptor supersensitivity. The results show that $FeCl_2$ (5 mg/kg per day for 21 days) given to rats treated with chlorpromazine (10 mg/kg per day, for 21 days) prevented the onset of supersensitive biochemical and behavioral (apomorphine) expressions of DA D_2 receptor. Inclusion of iron did not affect the chlorpromazine-induced sedation or hypothermia. Moreover, the combined chronic iron-chlorpromazine treatment produced the same net effects as chronic chlorpromazine on striatal amounts of dopamine, DOPAC (dihydroxyphenylacetic acid) and HVA (homovanillic acid). Chlorpromazine medication caused a decrease in liver non-haem iron levels (40%) but not in brain iron. The effect of the neuroleptic drug on iron stores and the involvement of iron in the neuroleptic-induced dopamine supersensitivity suggest that mobilization of iron from the periphery into the brain may play an important role in the mechanism of action of the neuroleptics.

Dopamine D_2 receptor supersensitivity; Chlorpromazine; Iron

1. Introduction

Tardive dyskinesia (TD) is the most severe side-effect of chronic neuroleptic treatment. The exact biochemical basis for it is obscure. However, dopamine D_2 receptor supersensitivity may be associated with the onset of TD (Klawans, 1973; Goetz et al., 1982). Previous studies have indicated the involvement of iron in modulation of dopamine D_2 receptors as demonstrated biochemically and behaviourally (Youdim, 1985; Ben-Shachar et al., 1985; Ben-Shachar and Youdim, 1987). Thus, injection of ferrous or ferric salts in rats, either into the amygdala-hippocampus complex or intraventricularly, caused a highly significant increase in dopamine D_2 receptor number in the striatum and in apomorphine-induced circling and hyperactivity (Csernansky et al., 1983; Ben-Shachar and Youdim, 1987). In contrast, nutritional iron deficiency in rats, which resulted in decreased brain iron levels (Dallman et al., 1975; Youdim and Green, 1977), was associated with dopamine D_2 receptor subsensitivity. This was evidenced by a significant and specific reduction in dopamine D_2 receptor B_{max}, together with a decrease in apomorphine-induced behaviours (Ashkenazi et al., 1982; Youdim et al., 1983).

Campbell et al. (1985) reported an extensive iron deposition in the substantia nigra, putamen, globus pallidus, caudate nucleus and subthalamic nucleus of patients with haloperidol-induced TD, confirming earlier results obtained by Hunter et al. (1968). In a matching animal study, Weiner et al. (1977) demonstrated the ability of chronic neuroleptic treatment to increase brain iron levels in the caudate nucleus of the guinea pig. These results may not be totally unexpected since the neuroleptics, at least the phenothiazines, are potent iron chelators (Borg and Cotzias, 1962a, b; Rajan et al., 1974). Moreover, chronic neuroleptic (phenothiazines and butyrophenones) treatment reduces liver iron levels in the rat (Ben-Shachar and Youdim, 1990). These findings support the theory that neuroleptics are able to affect tissue iron deposition by mobilizing iron stores. The mechanism of dopamine D_2 receptor supersensitivity as induced by neuroleptics thus remains obscure but its relationship to brain iron metabolism cannot be ignored.

The purpose of the present study was to investigate the interaction between iron and neuroleptic by exam-

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ining the effect of iron on chronic neuroleptic-induced dopamine D_2 receptor supersensitivity. Neuroleptic iron interaction attains greater significance if one considers the ability of iron to catalyze free radical formation and thereby potentiate neurodegeneration (Halliwell and Gutteridge 1986).

2. Materials and methods

2.1. Materials

[^{3}H]Spiperone (24-26 Ci/mmol) was purchased from New England Nuclear. Rat prolactin for iodination (r-PRL-B-6, 25 IU/mg), rPRL reference preparation (rPRL-RP-3; 30 IU/mg) and rabbit antiserum to rPRL (anti-rPRL-5-9) were provided by the National Hormone and Pituitary program of the NIADDK, National Institute of Health, Bethesda, MD, USA. Goat anti-rabbit IgG was purchased from Miles Yeda Ltd., Rehovot, Israel. Na^{125}I was purchased from Nuclear Research Center-Negev, Beer Sheva, Israel. Chlorpromazine-2HCl (CPZ) was a gift from Smith Kline and French Laboratory, Philadelphia division of Smith Kline Co. Haemoglobin kit, apomorphine, 2,4,6-tripyridyl-s-triazine (TPZ), dopamine homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), sodium octyl sulfate (SOS), 3,4-dihydroxybenzylamine, polyvinylpyrrolidone (PVP average molecular weight 360 000 Da) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company, and 1,10-phenanthroline from Merck. Other reagents were of highest purity and were purchased from standard commercial sources.

2.2. Animal treatment

Chlorpromazine (10 mg/kg) was injected (i.p.) together with or without ferrous chloride (5 mg/kg Fe^{2+}) daily for three weeks, to 21-day-old male Sprague Dawley rats. Another group received ferrous chloride (5 mg/kg Fe^{2+}) and the control group received saline. Following the last injection or after a 3-day drug free washout period the rats were either killed for biochemical determination or examined for their behavioural response to apomorphine.

2.3. Determination of non-haem iron and haemoglobin

The serum iron level was determined by the assay of Caraway (1963) using TPZ as an iron chelator. The haemoglobin level was measured using a commercial kit obtained from Sigma. Tissue non-haem iron was determined by a modification of Hallgren and Sourander (1958) and Weinfeld (1964) as described previously (Ben-Shachar et al., 1985).

2.4. Apomorphine-induced motor activity determination

Apomorphine (1 mg/kg i.p.) was injected to groups of two to three animals. Their locomotor activity was monitored on a Varimax activity meter for 30 min as described previously (Ben-Shachar et al., 1986).

2.5. [^{3}H]Spiperone binding

Specific [^{3}H]spiperone binding to caudate nucleus membrane homogenates (0.25 mg protein/0.4 ml of 0.05 M Tris-HCl buffer, pH 7.4) was determined as described previously (Briley and Langer, 1978; Ben-Shachar et al., 1985), [^{3}H]spiperone (0.05 ml; final concentrations 0.05-2 nM) was added in the presence or absence of 10 μM haloperidol for determination of non-specific binding, and in the presence of 10^{-6} M cinanserin to prevent labelling of 5-HT_2 sites by [^{3}H]spiperone. Incubation was carried out at 37 °C for 20 min. Incubation was terminated by rapid filtration, and the glass fibre filters (GF/B Whatman) were then counted for tritium content in a liquid scintillation counter.

2.6. Radioimmunoassay (RIAS) or rPRL

The PRL concentration in sera obtained from individual rats was measured by a double antibody method using reagents provided by the NIADDK as described previously (Barkey et al., 1985). All measurements were made in duplicate on 100-200 μl serum, taken from chronically treated rats 3 days after the last injection. The sensitivity of the assay was 0.1 ng/tube.

2.7. HPLC

The HPLC system (Model 5000 LC Varian, CA, USA) consisted of a 50×4.6 mm (inner diametre), 3-μm particle size Spherisorb S30DSII reversed phase column (Regis, IL, USA), a 10-μl sample injector, a model 5100 A Coulochem detector (ESA Inc., MA, USA) with model 5011 analytical cell and model 5020 guard cell. The dual electrode analytical cell operated in a redox mode with 0.3 V oxidation potential and -0.26 V reduction potential. Chromatographs were recorded at a detector sensitivity of 1 nA/V full scale.

The column was set at ambient temperature and the flow rate was 1.5 ml/min. The detector was connected to a Varian 4270 integrator (Varian, CA, USA) operated in a peak area mode. The mobile phase consisted of 28.3 g monochloroacetic acid, 9.35 g sodium hydroxide, 1.5 g disodium ethylendiamintetraacetic acid (Na_2 EDTA), 10.15 g sodium octyl sulfate and 10 ml methanol per liter and was adjusted to pH 3.0. The mobile phase was filtered through a 0.45-μm pore size

membrane filter (Gelman Sci. Inc.). The column was equilibrated with the mobile phase overnight.

Sample preparation: the rats were decapitated and the brains were rapidly removed. The caudate nucleus was separated on an ice-chilled glass plate and was quickly frozen in liquid nitrogen. Frozen tissues were weighed then homogenized in a glass homogenizer in 300 μl of 0.1 M perchloric acid (PCA) containing 15 ng of 3,4-dihydroxybenzyl amine (DBHA) as internal standard. After centrifugation at $30\,000 \times g$ for 15 min at 4 °C the clear supernatants were removed and assayed by HPLC.

2.8. Protein determination

Protein was determined using the method of Lowry et al. (1951).

2.9. Statistical analysis

All the data are expressed as means ± S.E.M. Statistical analysis was carried out by analysis of variance (ANOVA) with multiple comparisons, followed by two-tailed Student's t-test. P values represent a significant difference between the control and the matched treated groups.

3. Results

3.1. Physical effects of chlorpromazine and iron alone and in combination

CPZ (10 mg/kg i.p.) and a low dose of ferrous chloride (Fe^{2+}) (5 mg/kg i.p.), alone or in combination, given for 3 weeks to 21-day-old rats, did not elicit differences in weight gain as compared to saline-injected controls (table 1). Each injection of CPZ alone or of CPZ + Fe^{2+} caused immediate sedation. Moreover, there was a significant ($P < 0.001$) decreased in body temperature of the rats injected with CPZ and CPZ + Fe^{2+} as compared to the controls and to iron-injected rats 30 min after CPZ administration (fig. 1). These effects lasted for 10-12 h.

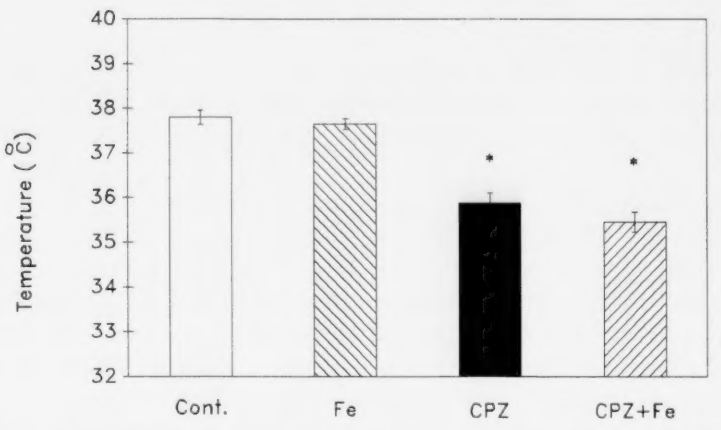

Fig. 1. The effect of concurrent chronic chlorpromazine (CPZ) and ferrous chloride (Fe) treatment on body temperature of the rat. Body temperature was measured each week. Representative results for the 10th day after the start of treatment are presented. Body temperature was measured before and 30 min after injection of CPZ (10 mg/kg i.p.) or Fe^{2+} (5 mg/kg i.p.) or the combination of both. The results are means ± S.E.M. or the body temperature of six rats in each group 30 min after the injection. The significant difference ($P < 0.01$) between groups was shown by ANOVA followed by a two-tailed Student's t-test. * $P < 0.001$.

3.2. Haemoglobin, serum iron, liver and brain non-haem iron

The haemoglobin levels were not affected by either the chronic neuroleptic or Fe^{2+} treatments. However, serum iron was reduced in the CPZ- and CPZ + Fe^{2+}-treated groups. Daily injection of 5 mg/kg $FeCl_2$ had no effect on serum iron (table 1), but increased liver non-haem iron levels when given separately or in combination with CPZ. While chronic treatment with CPZ caused a significant reduction in liver non-haem iron (table 1), brain non-haem iron levels were not affected by chronic iron, CPZ alone, or a combination of CPZ and iron (table 1).

TABLE 1

Iron status in rats injected chronically with ferrous chloride (Fe^{2+}), chlorpromazine (CPZ) or both.
21-day-old male rats were injected daily for 3 weeks with CPZ (10 mg/kg i.p.), ferric chloride (Fe^{2+} 5 mg/kg i.p.) or given the combined treatment. The values are means ± S.E.M. for 10-20 rats in each group. The significance of differences ($P < 0.01$) between groups for each parameter was shown by ANOVA. Subsequent comparison was carried out by two-tailed Student's t-test.

	Control	Fe^{2+}	CPZ	CPZ + Fe^{2+}
Body weight (g)	166 ± 6	162 ± 6	165 ± 3	164 ± 4
Haemoglobin (g%)	10.78 ± 0.24	11.44 ± 0.32	10.85 ± 0.49	11.6 ± 0.20 [a]
Serum iron (μg/g)	5.34 ± 0.28	5.70 ± 0.60	3.55 ± 0.45 [b]	4.13 ± 0.20 [b]
Liver non-haem iron (μg/g)	75.68 ± 9.60	160.00 ± 20.35 [b]	42.85 ± 7.95 [b]	196.90 ± 25.77 [b]
Caudate non-haem iron (μg/g)	43.75 ± 2.83	42.61 ± 3.60	48.91 ± 4.39	37.60 ± 2.76

[a] $P < 0.01$, [b] $P < 0.05$.

TABLE 2

Equilibrium constants for [^{3}H]spiperone binding to dopamine D_2 receptors and apomorphine-induced locomotor activity in rats treated chronically with ferrous chloride (Fe^{2+}) chlorpromazine (CPZ) or both.
21-day-old rats were injected daily for 3 weeks with CPZ (10 mg/kg i.p.) or Fe^{2+} (5 mg/kg i.p.) or both. Control rats received saline. The motor activity in response to apomorphine (2 mg/kg i.p.) was measured 2 days after the last injection, as described in Materials and methods. Three days after the last injection the rats were killed and binding of [^{3}H]spiperone was assayed in caudate nucleus membrane homogenates. Binding constants were calculated by computer fit. Saturation analysis was performed for eight concentrations of [^{3}H]spiperone (0.05-2 nM). The data are means ± S.E.M. values from four to five experiments three animals in each. The significance of differences ($P < 0.01$) between treatments was obtained by ANOVA. Subsequent comparisons between treatments and control were carried out by two-tailed Student's t-test.

	K_D (nM)	B_{max} (pmol/mg protein)	Apomorphine (movements/40 min)
Control	0.22 ± 0.018	0.32 ± 0.02	905 ± 46
Fe^{2+}	0.20 ± 0.024	0.36 ± 0.03	1012 ± 39
CPZ	0.26 ± 0.020	0.55 ± 0.06 [a]	1352 ± 42 [b]
CPZ + Fe^{2+}	0.19 ± 0.020	0.30 ± 0.04	1104 ± 100

[a] $P < 0.001$ ** $P < 0.005$.

3.3. [^{3}H]Spiperone binding to caudate nucleus membrane homogenates and apomorphine-induced behaviour

Chronic peripheral injection of $FeCl_2$ (5 mg/kg) had no effect on [^{3}H]spiperone binding to dopamine D_2 receptors in the striatum or on apomorphine-induced locomotor behaviour. Three weeks of daily injection of CPZ followed by 3 days of drug washout resulted in a significant ($P < 0.001$) increase in maximal number of [^{3}H]spiperone binding sites (B_{max}) in the caudate nucleus, but no change in the dissociation constant (K_D). Apomorphine-induced locomotor behaviour was also significantly ($P < 0.005$) increased. In contrast, the combined chronic treatment with CPZ and $FeCl_2$ failed to induce any change in B_{max} or in the affinity (K_D) or [^{3}H]spiperone to dopamine D_2 receptors in the caudate nucleus or to cause any change in apomorphine-induced locomotor activity (table 2). In the in vitro binding assay using caudate membrane homogenates, CPZ and CPZ + Fe^{2+} were equally potent to displace [^{3}H]spiperone. Thus 10^{-3}-10^{-9} M CPZ in the presence or absence of 10^{-4} or 5×10^{-4} M $FeCl_2$ displaced 1 nM [^{3}H]spiperone from caudate nucleus homogenates with an IC_{50} value of 80 nM.

TABLE 3

Serum PRL levels in rats injected chronically with ferrous chloride (Fe^{2+}), chlorpromazine (CPZ) or both.
Serum was collected from rats treated as described in table 2. The values are means ± S.E.M. for six to eight rats in each group. The significance of differences ($P < 0.01$) between treatments was obtained by ANOVA, followed by a two-tailed Student's t-test.

	Plasma rPRL (ng/ml)
Control	5.69 ± 1.50
Fe^{2+}	5.43 ± 1.57
CPZ	13.31 ± 2.60 [a]
CPZ + Fe^{2+}	5.66 ± 1.18

[a] $P < 0.05$.

3.4. Serum PRL levels

Serum PRL levels were not affected by chronic treatment with iron salts. Chronic CPZ treatment followed by 3 days of drug washout caused a 2-fold increase in serum PRL levels. The addition of iron salts to chronic CPZ treatment abolished the latter effect (table 3).

3.5. Dopamine, DOPAC and HVA concentrations in caudate nucleus

Chronic injection of CPZ or CPZ + Fe^{2+} for 3 weeks had no effect on dopamine levels but caused a significant ($P < 0.001$) decrease in HVA concentrations in the caudate nucleus. The observed decrease in DOPAC concentrations was not statistically significant. However, the tendency towards reduction was confirmed by the results for the DA/DOPAC ratio.

TABLE 4

Dopamine, DOPAC and HVA concentration in the caudate nucleus of rats treated chronically with ferrous chloride (Fe^{2+}), chlorpromazine (CPZ) or both.
Rats received the same treatment as described in table 1, except that they were killed after the last injection to prevent drug washout. The values are means ± S.E.M. of duplicate HPLC determinations for five rats in each group. The significance of differences ($P < 0.01$) between groups for each parameter was shown by ANOVA. Subsequent comparison was carried out by two-tailed Student's t-test.

	Concentrations (ng/mg tissue)				
	DA	DOPAC	HVA	DA/DOPAC	DA/HVA
Control	14.50 ± 2.00	2.16 ± 0.25	0.84 ± 0.006	6.71	17.26
Fe^{2+}	14.20 ± 0.95	2.28 ± 0.19	1.01 ± 0.14	6.23	14.06
Chlorpromazine	15.33 ± 0.40	1.70 ± 0.12	0.51 ± 0.06 [a]	9.02	30.06
Chlorpromazine + Fe^{2+}	14.90 ± 1.64	1.74 ± 0.14	0.53 ± 0.04 [a]	8.56	28.11

[a] $P < 0.001$.

Chronic ferrous chloride injection did not alter the levels of dopamine and its metabolites as compared to those in control rats (table 4).

4. Discussion

There is increasing evidence for a relationship between iron metabolism and chronic neuroleptic treatment. Weiner et al. (1987) found an increase in brain iron levels of guinea pigs treated chronically with chlorpromazine. Campbell et al. (1985) reported extensive iron deposition in the substantia nigra, putamen, globus pallidus, caudate nucleus and subthalamic nucleus of patients with haloperidol-induced TD, supporting earlier findings by Earl (1968) and Hunter et al. (1968). In the present study we have demonstrated that the combination of iron and CPZ given chronically to rats prevents the dopamine D_2 supersensitivity induced in rats by chronic CPZ treatment. This was true for dopamine D_2 B_{max} in the striatum as well as for apomorphine-induced locomotor activity. It is accepted that locomotor activity is regulated by the striatal dopaminergic system in the nucleus accumbens (Kelly et al., 1975). The present results indicate that iron effects on CPZ-induced dopaminergic supersensitivity are not restricted to the striatum. The alterations in serum prolactin levels further support this assumption. It is well established that dopamine directly suppresses the pituitary lactotrope and prolactin secretion into the portal circulation (for review see Lancrajan and Friesen, 1978) via the dopamine D_2 receptors. Chronic blockage of D_2 receptors by CPZ resulted in a significant increase of serum prolactin levels. However addition of the iron salt to the CPZ treatment prevented this effect, suggesting disinhibition of the pituitary dopaminergic input.

Dopamine turnover, an indicator of dopamine release, was decreased to the same extent by three weeks of continuous administration of CPZ or of CPZ plus Fe^{2+}. Both HVA and DOPAC concentrations in the striatum were decreased as a result of chronic neuroleptic treatment, confirming previous results (Seeman, 1986; Gunne et al., 1984). Dopamine levels however remained unchanged. Chronic peripheral injection of 5 mg/kg $FeCl_2$ alone had no net effect on any of these parameters. These findings are consistent with the limited ability of systemic iron to cross the blood-brain barrier (Dallman et al., 1977; Banks et al., 1988) and to affect central events. The mechanism by which chronic neuroleptic treatment induces a decrease in dopamine turnover is still unknown. One possible explanation could be that the supersensitivity of the postsynaptic receptor induces greater feedback inhibition of presynaptic dopamine release (Tarsy and Baldessarini, 1977). Depolarisation inactivation of the dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area could also contribute to this effect (Chiodo and Bunney, 1985). Other authors relate the decrease in dopamine turnover to the supersensitivity of the presynaptic autoreceptors which regulate dopamine release and synthesis (Nowak et al., 1983; Saller and Salama, 1985). The reduction in serum and liver non-haem iron due to chronic CPZ treatment may result from mobilization of iron from its stores by CPZ via chelation. Although chronic iron injection slightly increased liver iron stores, preliminary (unpublished data) histological results obtained with liver preparations showed no difference between normal and iron-treated rats with or without CPZ. In contrast with the previous findings of Weiner et al. (1987) for brains of guinea pigs, the levels of iron in rat brain were unaffected by the neuroleptic treatment. This may have been due to variations between species. The mechanism by which alteration of endogenous iron levels affects neuroleptic-induced dopamine D_2 receptor number variations remains to be elucidated. However, it may be related in part to the metal chelating ability of the neuroleptics (Borg and Cotzias, 1962a, b; Rajan et al., 1974). Thus iron levels may affect the equilibrium between the free neuroleptic drug which can bind to the dopamine D_2 receptor and the seemingly less effective iron-bound neuroleptic. This view is supported by the potentiation of dopamine receptor supersensitivity in iron deficient rats treated chronically with haloperidol or CPZ (Ben-Shachar and Youdim, 1990) and the blockade of chronic CPZ-induced supersensitivity by chronic iron treatment. The in vitro findings concerning the equal displacement capacity of both CPZ and CPZ + Fe^{2+} oppose this assumption. Thus, the ability to block dopamine D_2 receptors and initiate supersensitivity of the receptor may be mediated by two separate biochemical actions of the neuroleptic. The prevention of chronic neuroleptic-induced striatal dopamine D_2 receptor supersensitivity by chronic iron treatment does not appear to be due to changes in dopamine or its metabolite levels, excluding the possible involvement of a compensatory mechanism. The ability of iron to abolish the CPZ-induced increase in dopamine D_2 receptors, apomorphine-induced locomotor activity, as well as in serum prolactin levels, while maintaining the CPZ ability to induce sedation, hypothermia and the decrease in DA turnover, could suggest that iron reduces the neuroleptic concentration in the brain.

It is worth noting that, in iron deficiency, chronic haloperidol medication causes an extensive dopaminergic supersensitivity due to the increase in dopamine D_2 receptors B_{max}. In this case, the blood-brain barrier is selectively altered (opened) in the rat (Ben-Shachar et al., 1988). Thus, under these conditions, neuroleptics may be more accessible in the brain and there may

result a greater drug concentration and thereby an extensive dopamine D_2 receptor supersensitivity. On the other hand, binding of iron to the neuroleptic drug may increase its polarity and thereby reduce its ability to penetrate into the brain.

In conclusion, the role of an increased tissue iron content in preventing chronic neuroleptic-induced dopamine D_2 supersensitivity, without losing the therapeutic effectiveness of this class of drugs, may have considerable significance in treatment of schizophrenic patients and the appearance of TD. The mechanism by which availability of iron affects the permeability of the blood-brain barrier and prevents chronic neuroleptic-induced dopamine D_2 receptor supersensitivity is being further investigated.

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References

Ashkenazi, R., D. Ben-Shachar and M.B.H. Youdim, 1982, Nutritional iron and dopamine binding sites in the rat brain, Pharmacol. Biochem. Behav. 17 (Suppl.), 43.

Banks, W.A., A.J. Kastin, M.B. Fasold, C.M. Barrera and G. Augereau, 1988, Studies of the slow bidirectional transport of iron and transferrin across the blood brain barrier, Brain Res. Bull. 21, 881.

Barkey, R.J., D. Ben-Shachar, T. Amit and M.B.H. Youdim, 1985, Increased hepatic and reduced prostatic prolactin (PRL) binding in iron deficiency and during neuroleptic treatment: correlation with changes in serum PRL and testosterone, Eur. J. Pharmacol. 109, 193.

Ben-Shachar, D., R. Ashkenazi and M.B.H. Youdim, 1986, Long term consequences, of early iron deficiency on dopaminergic neurotransmission, Int. J. Dev. Neurosci. 4, 91.

Ben-Shachar, D., J.M.P. Finberg and M.B.H. Youdim, 1985, Effect of iron-chelators on dopamine D_2 receptors, J. Neurochem. 45, 999.

Ben-Shachar, D., S. Yehuda, J.M.P. Finberg, I. Spanier and M.B.H. Youdim, 1988, Selective alteration in blood brain barrier and insulin transport in iron deficient rats, J. Neurochem. 50, 1434.

Ben-Shachar, D. and M.B.H. Youdim, 1987, Neuroleptic induced dopamine receptor supersensitivity and tardive dyskinesia may involve altered brain iron metabolism, Br. J. Pharmacol. 90, 95P.

Ben-Shachar, D. and M.B.H. Youdim, 1990, Neuroleptic induced supersensitivity and brain iron: I. Iron-deficiency and neuroleptic induced dopamine D_2 receptor supersensitivity, J. Neurochem. 54, 1136.

Borg, D.G. and G.C. Cotzias, 1962a, Interaction of trace metal with phenothiazine drug derivatives. III Theoretical part, Proc. Natl. Acad. Sci. U.S.A. 48, 617.

Borg, D.G. and G.C. Cotzias, 1962b, Interaction of trace metal with phenothiazine drug derivatives. II. Formation of free radicals, Proc. Natl. Acad. Sci. U.S.A. 48, 623.

Briley, M. and S.Z. Langer, 1978, Two binding sites for [^{3}H]-spiroperidol on rat striatal membranes, Eur. J. Pharmacol. 50, 283.

Campbell, W.G., M.A. Raskind, T. Gordon and C.M. Shaw, 1985, Iron pigment in the brain of a man with tardive dyskinesia, Am. J. Psychiat. 142, 365.

Caraway, W.T., 1963, Macro and micro method for the determination of serum iron and iron binding capacity, Clin. Chem. 9, 188.

Chiodo, L.A. and B.S. Bunney, 1985, Possible mechanism by which repeated clozapine administration differentially affects the activity of two subpopulations of midbrain neurons, J. Neurosci. 5, 2539.

Csernansky, J.C., C.A. Holman, K.A. Bounet, K. Grabaowsky, R. King and L.E. Hollister, 1983, Dopaminergic supersensitivity at distant sites following induced epileptic foci, Life Sci. 32, 385.

Dallman, P.R., M.A. Siimes and E.C. Manies, 1975, Brain iron persistent deficiency following short-term iron deprivation in young rats, Br. J. Haematol. 31, 205.

Dallman, P.R. and R.A. Spirito, 1977, Brain iron in the rat: extremely slow turnover in normal rat may explain long-lasting effects of early iron deficiency, J. Nutrit. 107, 1075.

Earl, K.M., 1968, Trace metals in parkinsonian brain, J. Neuropathol. Exp. Neurol. 27, 1.

Goetz, C.G., W.J. Weiner, P.A. Nausieda and H.L. Klawans, 1982, Tardive dyskinesia: pharmacology and clinical implications, Clin. Neuropharmacol. 5, 3.

Gunne, L.M., J.E. Hagström and B. Sjöquist, 1984, Association with persistent neuroleptic induced dyskinesia of regional changes in brain GABA synthesis, Lett. Nature 309, 347.

Hallgren, B. and P. Sourander, 1958, The effect of age on the nonhemin iron in the human brain, J. Neurochem. 3, 41.

Halliwell, B. and M.C. Gutteridge, 1986, Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts, Arch. Biochem. Biophys. 246, 501.

Hunter, R., W. Blackwood and M.C. Smith, 1968, Neuropathological findings in three cases of persistent dyskinesia following phenothiazine medication, J. Neurol. Sci. 7, 763.

Kelly, P.H., P.W. Seviour and S.D. Iversen, 1975, Amphetamine and apomorphine responses in the rat following 6-OHDA lesions of the nucleus accumbens septi and corpus striatum, Brain Res. 94, 505.

Klawans, H.L., 1973, The pharmacology of tardive dyskinesia associated with metoclopranide, Br. Med. J. 1, 77.

Lancrajan, I. and H.G. Friesen, 1978, The neural regulation of prolactin secretion, in: Current Studies of Hypothalamic Function, Vol. 1, eds. W.L. Veale and K. Ledems (Karger, Basel) p. 131.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Nowak, J.Z., S. Arbilla, A.M. Galzin and S.Z. Langer, 1983, Changes in sensitivity of release modulating dopamine autoreceptors after chronic treatment with haloperidol, J. Pharmacol. Exp. Ther. 226, 558.

Rajan, K.S., A.A. Manian, J.M. Davis and R.L. Nixon, 1974, Studies on metal chelation of chlorpromazine and its hydroxylated metabolites, Adv. Biochem. Psychopharmacol. 9, 571.

Saller, C.H. and A.I. Salama, 1985, Alterations in dopamine metabolism after chronic treatment with haloperidol, J. Pharmacol. Exp. Ther. 226, 558.

Seeman, P., 1987, Dopamine receptors and dopamine hypothesis of schizophrenia, Synapse 1, 133.

Tarsy, D. and R.J. Baldessarini, 1977, The pathophysiologic bases of tardive dyskinesia, Biol. Psychiat. 12, 431.

Weiner, W.J., P.A. Nausieda and H.J. Klawans, 1977, Effect of chlorpromazine on central nervous system concentrations of manganese, iron and copper, Life Sci. 20, 1181.

Weinfeld, A., 1964, Storage iron in man, Acta Med. Scand. 177, (Suppl. 427), 1.

Youdim, M.B.H., 1985, Brain iron metabolism biochemical and behavioural aspects in relation to dopaminergic neurotransmission, in: Handbook of Neurochemistry, Vol. 10, ed. A. Lajath (Plenum Press, New York) p. 637.

Youdim, M.B.H., D. Ben-Shachar, R. Ashkenazi and S. Yehuda, 1983, Modulation of dopamine receptor in striatum by iron: behavioural and biochemical correlates, in: Advances in Neurology, Vol. 40, eds. A. Hassler and C.J. Christ (Raven Press, New York) p. 159.

Youdim, M.B.H., A.R. Green, M. Bloomfield, B. Mitchell, D.J. Heal and D.G. Graham-Smith, 1979, The effects of iron deficiency on brain biogenic monoamine biochemistry and function in rats, Neuropharmacology 19, 259.

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5-HT_2 presynaptic receptors mediate inhibition of glutamate release from cerebellar mossy fibre terminals

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'Giant' synaptosomes originating from mossy fibre terminals and having sedimentation properties different from those of standard synaptosomes were obtained from rat cerebellum. Exposure of superfused giant synaptosomes to 15 mM KCl caused the release of endogenous glutamate in a largely (about 80%) calcium-dependent manner. The K^+-evoked overflow of glutamate was inhibited in a concentration-dependent manner by 5-hydroxytryptamine (5-HT) and by the 5-HT_2 receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI), but not by the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT). The effects of 5-HT and DOI were quite potent, already reaching significant inhibition (about 25%) at 10 nM. The 5-HT_2 receptor antagonist ketanserin counteracted the inhibitory effect of 5-HT. In cerebellar slices, ketanserin increased on its own the calcium-dependent K^+-evoked release of glutamate and this effect was not prevented by tetrodotoxin (TTX). The results support the idea that cerebellar mossy fibres use glutamate as a transmitter and show that the release of glutamate can be inhibited via presynaptic heteroreceptors of the 5-HT_2 type probably localized on the mossy fibre terminals.

Mossy fibres; Cerebellum (rat); Glutamate release; 5-HT_2 receptors (presynaptic); Synaptosomes; (Slices)

1. Introduction

It has been found previously that 5-hydroxytryptamine (5-HT) potently inhibits the release of endogenous glutamate from rat cerebellar slices depolarized with high K^+. Interestingly, the inhibition is mediated by two types of serotonin receptors: 5-HT_1-like and 5-HT_2 (Maura et al., 1988). Studies with cerebellar synaptosomes have shown that the 5-HT_1-like receptors are sited on glutamate-releasing axon terminals, while the receptors of the 5-HT_2 type appear to be located on structures which are lost during the standard preparation of synaptosomes (Raiteri et al., 1986; Maura et al., 1988).

It has been reported that during homogenization of cerebellar tissue two types of synaptosomes are produced: standard synaptosomes and 'giant' synaptosomes. The latter appear to originate from the endings of mossy fibres. Because of the different sedimentation properties, giant synaptosomes are not present in standard synaptosomal preparations. However, they can be obtained from the nuclear fraction of cerebellar homogenates (Israel and Whittaker, 1965; Hajós et al., 1975).

Mossy fibres make excitatory synaptic connections with granule cells (Ito, 1984). According to Somogyi et al. (1986), glutamate immunoreactivity is concentrated in mossy fibre terminals of the cat cerebellum. Moreover, the results of a recent electrophysiological study 'suggest that an excitatory amino acid, presumably glutamate, is the major mossy fibre transmitter in the cerebellum' (Garthwaite and Brodbelt, 1990).

The above considerations prompted us to investigate if mossy fibre endings could be the site where the 5-HT_2 receptors mediating inhibition of glutamate release in cerebellar slices are located.

2. Materials and methods

Adult male rats (Wistar 200-250 g) were killed by decapitation. The cerebellum was rapidly removed from the skull and placed in ice-cold medium.

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2.1. Experiments with giant synaptosomes

The giant synaptosomes were prepared according to Israel and Whittaker (1965) with some modifications. The cerebellum was removed and homogenized in 40 volumes of 0.32 M sucrose buffered with phosphate at pH 7.4. The homogenate was centrifuged at $1000 \times g$ for 5 min. The pellet (P_1; crude nuclear fraction) was resuspended in an equal volume of sucrose 0.32 M pH 7.4, filtered through a double gauze layer, and then centrifuged at $1000 \times g$ for 5 min. The pellet containing the giant synaptosomes was resuspended in a physiological salt solution of the following composition (mM): NaCl 125; KCl 3; $MgSO_4$ 1.2; $CaCl_2$ 1.2; NaH_2PO_4 1.0; $NaHCO_3$ 22 and glucose 11; pH 7.2-7.4; gassed with 95% O_2 and 5% CO_2 at 37 °C. Giant synaptosomes were incubated 15 min at 37 °C in standard medium. After incubation, aliquots of the synaptosomal suspension were distributed at the bottom of a set of parallel superfusion chambers maintained at 37 °C (Raiteri et al., 1974). Superfusion was started at a rate of 0.6 ml/min with standard medium aerated continuously with 5% CO_2 in O_2. Glutamate release was evoked by exposure to 15 mM KCl (replacing an equimolar concentration of NaCl) for 120 s, after 38 min superfusion. Two 3-min samples (basal outflow) were collected before and after the 6-min sample containing the glutamate released by the depolarization pulse. The superfusate fractions were collected in plastic vials and rapidly frozen. The amount of glutamate released into each fraction is expressed as pmol per mg of protein (protein determination was carried out according to Bradford, 1976). Agonists were added concomitantly with high K^+, and antagonists 8 min before depolarization. The K^+-evoked overflow was calculated by subtraction of the pmoles of glutamate present in the two 3-min fractions (pre- and poststimulation basal release) from the 6-min fractions corresponding to the high K^+ stimulation.

2.2. Experiments with cerebellar slices

The isolated cerebellum was chopped, using a McIlwain tissue chopper, in a sagittal plane into 250 μm slices weighing 6.7 ± 0.57 mg (mean $\pm$ S.E.M.; $n = 15$). The slices were incubated for 10 min in physiological salt solution (see 2.1.). After incubation, 8-12 slices were transferred to the bottom of parallel superfusion chambers maintained at 37 °C (see 2.1.). Superfusion was started at a rate of 0.6 ml/min with standard medium aerated continuously with CO_2 in O_2; duration 76 min. The superfusate was collected in 3-min samples (basal outflow) before and after 6-min samples (evoked release) had been collected. Two 2-min periods of depolarization (35 mM KCl replacing an equimolar concentration of NaCl) were applied 38 and 66 min after the start of superfusion (S_1 and S_2, respectively). The superfusate fractions were collected in plastic tubes and rapidly frozen. The amount of glutamate released into each fraction was determined and is expressed as pmol/fraction or as a percentage of the total tissue glutamate content at the start of the fraction considered. The K^+-evoked overflow (S_1 or S_2) was calculated by subtracting the pmol or the percentage of glutamate present in the two 3-min fractions collected before and after the stimulation from the total glutamate content in the 6-min fraction corresponding to S_1 or S_2. When used, tetrodotoxin (TTX) was added to the superfusion medium 18 min before S_1 and was present throughout the experiment; ketanserin was added 8 min before S_2.

The effect of ketanserin on the K^+-evoked overflow was measured as the percent variation of the S_2/S_1 ratio with respect to the control value, i.e. (S_2/S_1 for drug-treated slices)/(S_2/S_1 for control slices) $\times 100$.

2.3. Glutamic acid determination

The amount of endogenous glutamic acid released from or remaining in the slices or in the giant synaptosomes after superfusion was measured by high-performance liquid chromatography (HPLC). The glutamate content of slices or giant synaptosomes was measured in the supernatant obtained after homogenization (Ultra Turrax maximal speed, 20 s) in ice-cold distilled water and centrifugation at $20\,000 \times g$ for 10 min. The analytical method involved precolumn derivatization with o-phthalaldehyde followed by separation on a C_{18} reverse-phase chromatography column with fluorescence detection according to Tonnaer et al. (1983). The retention time of glutamate in the analysed samples was identical to that of authentic glutamic acid tested regularly once every four to five samples. HPLC was performed with a Perkin Elmer series 2 and a Rheodyne 6125 injector. The column effluent was monitored with a Shimadzu RF 535 fluorescence HPLC monitor. The mobile phase was 0.05 mM phosphate buffer, pH 5.5, and 27% methanol (Lichrosolv, Merck & Co. Inc. Rahway, NJ).

2.4. Electron microscopy

Suspensions of giant synaptosomal fractions were fixed for 2 h with 1% glutaraldehyde and 3% paraformaldehyde in 0.2 M phosphate buffer (PB), pH 7.4, at 4 °C, pelleted and washed in the same buffer. Then 0.5 ml aliquots of fixed synaptosomes were mixed with an identical volume of 3% agarose in PB at 54 °C. Immediately afterwards, small amounts of the agarose-suspension were placed in a plastic mold and allowed to cool and solidify. Small squares of the synaptosome-containing agarose films were washed

with PB, postfixed for 1 h in OsO_4 at 4 °C, quickly washed in PB, dehydrated and embedded in Durcupan (Fluka). Ultrathin sections were then cut with a Reichert ultramicrotome and stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Philips 301 electron microscope.

2.5. Statistical analysis

Means ± S.E.M. of the number of experiments (n) are reported throughout. Student's t-test was used to analyse the significance of the difference between two means.

2.6. Chemicals

The following drugs were purchased: 5-hydroxytryptamine creatinine sulphate (5-HT) (Calbiochem, Los Angeles, CA), 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) (RBI, Natick, MA); TTX (Sigma Co., St. Louis, MO). Ketanserin tartrate was a gift (Janssen, Beerse, Belgium) and was dissolved in 100 μl of glacial acetic acid and diluted to 1 mM in distilled water. The other compounds were dissolved in water to 1 mM. Final dilutions were made with physiological salt solution.

3. Results

3.1. Giant cerebellar synaptosomes

Figure 1 shows a typical electron microscope profile of a giant synaptosome prepared from the rat cerebellum. The average diameter of giant synaptosomes present in the preparation used in this study was 2.5-3 μm. The diameter of standard synaptosomes has been reported to be about 0.5 μm (Israel and Whittaker, 1965).

When giant synaptosomes from rat cerebellum were exposed in superfusion to 15 mM KCl for 120 s, the release of endogenous glutamate was increased over the basal outflow by more than 200%. Expressed as a percentage of the total synaptosomal content, the glutamate found in the 3-min samples collected before and after K^+ stimulation amounted to 0.53 ± 0.04/min (n = 5) and to 0.43 ± 0.04/min (n = 5), respectively. The K^+-evoked overflow (6-min sample) was 9.21 ± 0.78% (n = 5). The glutamate content in the 3-min sample collected before K^+ stimulation amounted to 118 ± 10.7 pmol/mg synaptosomal protein (n = 8); after stimulation it was 98 ± 0.79 pmol/mg protein (n = 8), while the K^+-evoked overflow (6-min sample) amounted to 438 ± 49.3 pmol/mg protein (n = 8). The depolarization-evoked release of the excitatory amino acid was largely dependent (about 80%) on the presence of calcium ions in the superfusion medium (table 1).

Fig. 1. Electron micrograph of a giant synaptosome present in a preparation from the rat cerebellum. The light synaptoplasm contains clearly outlined synaptic vesicles and two mitochondria. Glutaraldehyde-paraformaldehyde fixation; Durcupan embedding and uranyl acetate-lead citrate staining, ×30000.

Exogenous 5-HT (10-1000 nM), added concomitantly with the depolarizing stimulus during superfusion, inhibited the K^+-evoked release of glutamate in a concentration-dependent manner (fig. 2). The effect of

TABLE 1

Depolarization-evoked release of endogenous glutamate from cerebellar giant synaptosomes and its calcium dependence. Synaptosomes were incubated 15 min at 37 °C and superfused as described under Materials and methods. Depolarization was carried out with 15 mM KCl in either standard medium or medium from which calcium had been omitted 8 min before depolarization. Fractions of superfusate were collected and analysed by HPLC. The values represent means ± S.E.M. of six to eight separate experiments in duplicate.

	Endogenous glutamate (pmol/mg protein)	
	Standard medium	Ca^{2+}-free medium
Basal release [a]	216 ± 18.7	200 ± 20.8
K^+-evoked release	658 ± 52.3	301 ± 28.0

[a] Total amount in the two 3-min fractions collected before and after the 6-min fraction corresponding to K^+ depolarization (see Methods).

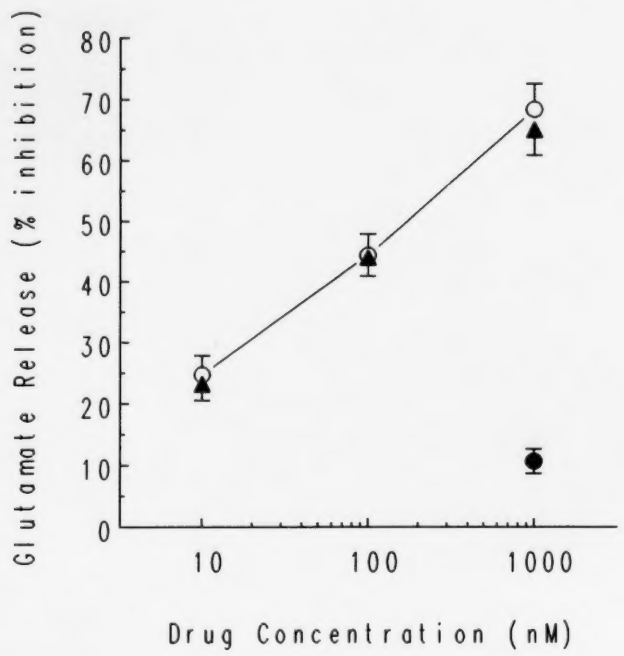

Fig. 2. Effect of 5-HT (○), DOI (▲) or 8-OH-DPAT (●) on the K^+ (15 mM)-evoked release of endogenous glutamate from rat cerebellar giant synaptosomes. Agonists were added to the superfusion medium concomitantly with 15 mM KCl. Experimental details as in Materials and methods. Each point represents the mean ± S.E.M. of three to five experiments in duplicate.

the indoleamine was already significant (25% inhibition) at a concentration as low as 10 nM. The 5-HT_2 receptor agonist DOI mimicked the inhibitory effects of 5-HT. In contrast, the 5-HT_{1A} receptor agonist 8-OH-DPAT, tested at 1000 nM, produced only a slight inhibition of glutamate release (fig. 1).

The inhibitory effect of 5-HT on the K^+-evoked release of glutamate from giant cerebellar synaptosomes was prevented by the selective 5-HT_2 receptor

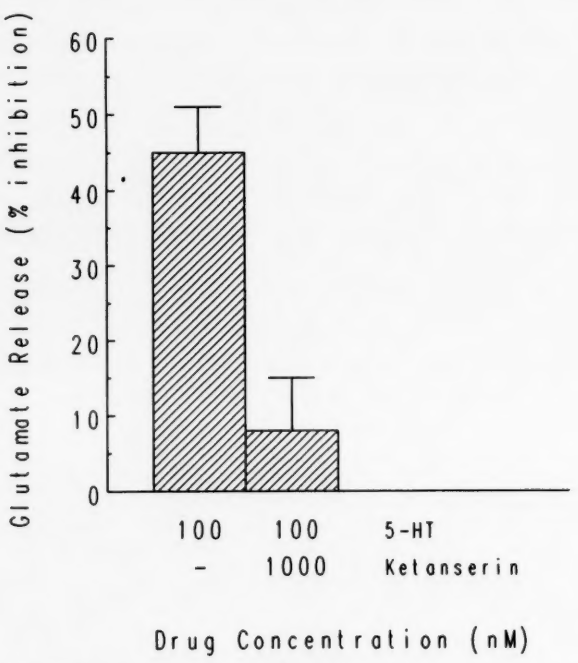

Fig. 3. Effect of ketanserin on the inhibition by 5-HT of the K^+ (15 mM)-evoked release of endogenous glutamate from rat cerebellar giant synaptosomes. Exogenous 5-HT was added concomitantly with high K^+ and the antagonist was present from 8 min before depolarization. Other experimental details as in Methods. Means ± S.E.M. of four to five experiments in duplicate are presented.

TABLE 2

Effects of ketanserin on the Ca^{2+}-dependent K^+-evoked release of endogenous glutamate from slices of rat cerebellum in the absence or presence of TTX. Slices were stimulated with high K^+ twices at min 38 (S_1) and at min 66 (S_2) of superfusion. S_2 was applied in either standard medium or medium from which Ca^{2+} had been omitted 8 min before depolarization. Ketanserin was added 8 min before S_2. TTX was present from 18 min before S_1 until the end of the experiment. For other experimental details see Methods. Means ± S.E.M. of three to four separate experiments in duplicate are shown.

	S_2/S_1 ratio
35 mM KCl	0.88 ± 0.07
35 mM KCl (Ca^{2+}-free)	0.07 ± 0.003
35 mM KCl + 1 μM ketanserin	1.11 ± 0.09 [a]
35 mM KCl + 1 μM TTX	0.86 ± 0.07
35 mM KCl + 1 μM ketanserin + 1 μM TTX	1.29 ± 0.1 [a]

[a] Significantly different ($P < 0.05$) from the respective control values.

antagonist ketanserin (fig. 3). Given alone, this drug did not affect the release of glutamate from the synaptosomal preparation under the experimental conditions used (data not shown).

3.2. Cerebellar slices

In rat cerebellar slices the basal outflow of glutamate, expressed as a percentage of the total tissue content, was 0.14 ± 0.01/min in the 3-min fraction before S_1 and 0.08 ± 0.01/min before S_2 (n = 4). The overflow of glutamate evoked by S_1 and by S_2 amounted to 2.41 ± 0.21 and 2.11 ± 0.19 (n = 4), respectively. Omission of calcium from the superfusion medium 8 min before S_2 decreased the K^+-evoked overflow by about 90%.

The calcium-dependent overflow of endogenous glutamate evoked by 35 mM KCl was increased by ketanserin (1 μM). This effect was TTX-resistant (table 2).

4. Discussion

Serotonergic fibres arising from the raphe nuclei (Chan-Palay, 1976, 1977; Shinnar et al., 1975) and from the nucleus reticularis gigantocellularis, the nucleus paragigantocellularis and the nucleus pontis oralis of the reticular formation (Bishop and Ho, 1985) are distributed throughout the cerebellum (Hökfelt and Fuxe, 1969; Chan-Palay, 1975, 1977; Bobillier et al., 1976; Takeuchi et al., 1982).

One important function of the 5-HT released from these fibres seems that of exerting an inhibitory control on the excitatory transmission mediated by glutamate and/or aspartate. This notion is based on the finding that activation of 5-HT receptors in the cerebellum leads to potent inhibition of the calcium-dependent

release of endogenous excitatory amino acids in depolarized slices or synaptosomes (Raiteri et al., 1986; Maura et al., 1988, 1991).

It is well known that 5-HT receptors are highly heterogeneous and have been subdivided into three types: 5-HT_1-like (further subdivided into several subtypes), 5-HT_2 and 5-HT_3 (Bradley et al., 1986; Frazer et al., 1990). The serotonergic inhibition of glutamate release from cerebellar slices is mediated by receptors of both the 5-HT_1-like and the 5-HT_2 type (Raiteri et al., 1986; Maura et al., 1988). Experiments with rat cerebellar synaptosomes have shown that 5-HT_1-like receptors (sensitive to 8-OH-DPAT and therefore similar, although not identical, to the 5-HT_{1A} subtype) are sited on glutamate-releasing axon terminals (Raiteri et al., 1986). In contrast, receptors of the 5-HT_2 type appear not to be present on isolated nerve endings but to be localized on structures which are lost during the standard preparation of synaptosomes.

It has been reported (Israel and Whittaker, 1965, Hajós et al., 1975) that during homogenization of cerebellar tissue not only 'standard' synaptosomes are formed but also giant synaptosomes originating from mossy fibre endings. Because of their different sedimentation characteristics, standard synaptosomes apparently do not contain the giant relatives which, on the other hand, can be recovered and partially purified from the nuclear fraction of the original homogenate. Immunohistochemical, electrophysiological and pharmacological evidence indicates that glutamate is the major mossy fibre transmitter (Beitz et al., 1986; Somogyi et al., 1986; Garthwaite and Brodbelt, 1990). Thus the possibility exists that the 5-HT_2 receptors mediating inhibition of glutamate release in cerebellar slices (but not in standard cerebellar synaptosomes) are located on mossy fibre endings.

The data of table 1, showing that giant synaptosomes prepared from rat cerebellum release endogenous glutamate in a calcium-dependent way when exposed in superfusion to a depolarizing stimulus, are in keeping with the idea that glutamate is a mossy fibre transmitter.

Nanomolar concentrations of 5-HT inhibited the calcium-dependent depolarization-evoked release of endogenous glutamate from superfused giant synaptosomes. The action of 5-HT was mimicked by the 5-HT_2 receptor agonist DOI but not by the 5-HT_{1A} receptor agonist 8-OH-DPAT. Ketanserin, a 5-HT_2 receptor blocker, antagonized the action of 5-HT. Thus the inhibition of glutamate release brought about by 5-HT appears to be mediated by receptors of the 5-HT_2 type. Moreover, considering that the superfusion technique used minimizes indirect effects, the 5-HT_2 receptors are likely to be sited on the giant synaptosomes.

The minimal activity of 8-OH-DPAT (fig. 2) indicates that contamination by glutamatergic synaptosomes carrying 5-HT_1-like receptors (Raiteri et al., 1986) was quite low. Reciprocally, giant synaptosomes appear not to be present in the standard synaptosomal preparations studied previously (Raiteri et al., 1986). Thus the difference in the sedimentation properties of the two types of cerebellar glutamatergic axon terminals seems so remarkable that a complete separation can be achieved by a relatively simple procedure.

Ketanserin increased the K^+-evoked release of endogenous glutamate in rat cerebellar slices. This effect could be interpreted as being due to disinhibition of the release that had been inhibited by the endogenous transmitter present in the biophase of the 5-HT_2 presynaptic heteroreceptor. According to the general view, this finding may indicate that the receptor in question plays a physiological role.

Because of the peculiarity of the synaptosomal preparation used, we thought that we need to verify the localization of the 5-HT_2 receptors on giant nerve terminals. TTX is often used in K^+-depolarized slices to ascertain whether the presynaptic modulation of release is direct (TTX-resistant) or indirect (TTX-sensitive). The finding that the enhancing effect of ketanserin on the K^+-evoked release of glutamate in cerebellar slices was not affected by TTX (table 2) strengthens the idea that 5-HT_2 receptors are located presynaptically, most likely on the mossy fibres nerve terminals.

On the basis of the literature and of our results, the following conclusions can be drawn: (a) as previously proposed by various authors (Beitz et al., 1986; Somogyi et al., 1986; Garthwaite and Brodbelt, 1990), glutamic acid is probably a mossy fibre transmitter in the cerebellum; (b) assuming that giant synaptosomes represent mossy fibre terminals, the release of glutamic acid from these fibres can be potently inhibited by 5-HT; (c) this effect is mediated by receptors of the 5-HT_2 type sited on the mossy fibre terminals themselves; (d) cerebellum serotonergic afferents appear to make direct synaptic contact with the mossy fibre terminals; (e) the release of glutamate from standard cerebellar synaptosomes can also be inhibited by 5-HT acting on receptors of the 5-HT_1-like type (Raiteri et al., 1986) that are possibly localized on the parallel fibre and climbing fibre terminals and varicosities (Zhang et al., 1990).

The potent inhibitory action of 5-HT on cerebellar glutamate release may have practical implications. It has been reported that administration of 5-hydroxytryptophan, the precursor of 5-HT, leads to improvement of human cerebellar ataxias (Trouillas et al., 1988). Increasing 5-HT formation might help to correct inappropriately elevated levels of this excitatory amino acid transmitter through the activation of presynaptic 5-HT_1-like or/and 5-HT_2 heteroreceptors. In this case, drugs selective for these receptors or able to increase

directly (5-HT releasers) or indirectly (5-HT re-uptake inhibitors) the concentration of the indoleamine in the biophase of these receptors may represent alternative therapeutic approaches.

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References

Beitz, A.J., A.A. Larson, P. Monaghan, R.A. Altschuler, M.M. Mullett and J.E. Madl, 1986, Immunohistochemical localization of glutamate, glutaminase and aspartate aminotransferase in neurons of the pontine nuclei of the rat, Neuroscience 17, 741.

Bishop, G.A. and R.H. Ho, 1985, The distribution and origin of serotonin immunoreactivity in the rat cerebellum, Brain Res. 331, 195.

Bobillier, P., S. Seguin, F. Petitjean, D. Salvert, M. Touret and M. Jouvet, 1976, The raphe nuclei of the cat brain stem: A topographical atlas of their efferent projections as revealed by autoradiography, Brain Res. 113, 449.

Bradford, M.M., 1976, Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 246.

Bradley, P.B., G. Engel, W. Feniuk, J.R. Fozard, P.P.A. Humphrey, D.N. Middlemiss, E.J. Mylecharane, B.P. Richardson and P.R. Saxena, 1986, Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine, Neuropharmacology 25, 563.

Chan-Palay, V., 1975, Fine structure of labelled axons in the cerebellar cortex and nuclei of rodents and primates after intraventricular infusions with tritiated serotonin, Anat. Embryol. 148, 235.

Chan-Palay, V., 1976, Serotonin afferents from raphe nuclei to the cerebellum in mammals, Exp. Brain Res. (Suppl.) 1, 20.

Chan-Palay, V., 1977, The indoleamine afferent axons to the cerebellum, in: Cerebellar Dentate Nucleus: Organization, Cytology and Transmitters (Springer, New York) p. 390.

Frazer, A., S. Maayani and B.B. Wolfe, 1990, Subtypes of receptors for serotonin, Ann. Rev. Pharmacol. Toxicol. 30, 307.

Garthwaite, J. and A.R. Brodbelt, 1990, Glutamate as the principal mossy fibre transmitter in rat cerebellum: pharmacological evidence, Eur. J. Neurosci. 2, 177.

Hajós, F., G. Wilkin, J. Wilson and R. Balazs, 1975, A rapid procedure for obtaining a preparation of large fragments of the cerebellar glomeruli in high purity, J. Neurochem. 24, 1277.

Hökfelt, T. and K. Fuxe, 1969, Cerebellar monoamine nerve terminals, a new type of afferent fibers to the cortex cerebelli, Exp. Brain Res. 9, 63.

Israel, M. and V.P. Whittaker, 1965, The isolation of mossy fibre endings from the granular layer of the cerebellar cortex, Experientia XXI, 325.

Ito, M., 1984, The Cerebellum and Neural Control (Raven Press, New York).

Maura, G., E. Roccatagliata, M. Ulivi and M. Raiteri, 1988, Serotonin-glutamate interaction in rat cerebellum: involvement of 5-HT_1 and 5-HT_2 receptors, Eur. J. Pharmacol. 145, 31.

Maura, G., A. Barzizza, S. Folghera and M. Raiteri, 1991, Release of endogenous aspartate from rat cerebellum slices and synaptosomes: inhibition mediated by a receptor of the 5-HT_2 type and by a 5-HT_1 receptor of a possibly novel subtype, Naunyn-Schmiedeb. Arch. Pharmacol. 343, 229.

Raiteri, M., F. Angelini and G. Levi, 1974, A simple apparatus for studying the release of neurotransmitters from synaptosomes, Eur. J. Pharmacol. 25, 411.

Raiteri, M., G. Maura, G. Bonanno and A. Pittaluga, 1986, Differential pharmacology and function of two 5-HT_1 receptors modulating transmitter release in rat cerebellum, J. Pharmacol. Exp. Ther. 237, 644.

Shinnar, S., R.J. Maciewicz and R.J. Shofer, 1975, A raphe projection to cat cerebellar cortex, Brain Res. 97, 139.

Somogyi, P., K. Halasy, J. Somogyi, J. Storm Mathisen and O.P. Ottersen, 1986, Quantification of immunogold labelling reveals enrichment of glutamate in mossy and parallel fibre terminals in cat cerebellum, Neuroscience 19, 1045.

Takeuchi, Y., H. Kimura and Y. Sano, 1982, Immunohistochemical demonstration of serotonin-containing nerve fibers in the cerebellum, Cell Tissue Res. 226, 1.

Tonnaer, J.A.D.M., G.M.H. Engels, V.M. Wiegant, J.P.H. Burbach, W. De Jong and D. De Wied, 1983, Proteolytic conversion of angiotensins in rat brain tissue, Eur. J. Biochem. 131, 415.

Trouillas, P., F. Brudon and P. Adeleine, 1988, Improvement of cerebellar ataxia with levorotatory form of 5-hydroxytryptophan. A double-blind study with quantified data processing, Arch. Neurol. 45, 1217.

Zhang, N., F. Walberg, J.H. Laake, B.S. Meldrum and O.P. Ottersen, 1990, Aspartate-like and glutamate-like immunoreactivities in the inferior olive and climbing fibre system: a light microscopic and semiquantitative electron microscopic study in rat and baboon (*Papio Anubis*), Neuroscience 38, 61.

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Role of dopamine D_1 and D_2 receptors in mediating the d-amphetamine discriminative cue

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The role of D_1 and D_2 dopamine (DA) receptors in mediating the discriminative cue produced by d-amphetamine (0.5 mg/kg) in rats has been assessed by using compounds which exert strong selectivity for each of these DA receptor subtypes. The D_2 agonists quinpirole and RU 24213 substituted completely for d-amphetamine, while the D_1 agonists SKF 38393 and SKF 81297 failed to exert such effects. On the other hand, the D_2 antagonists raclopride and YM 09151-2, and D_1 antagonists SCH 23390 and SKF 83566, all completely blocked d-amphetamine discrimination. The D_2 antagonists produced more pronounced inhibitory effects on response rate than did D_1 antagonists. Quinpirole substitution for d-amphetamine was blocked by YM 09151-2, but not by SCH 23390, while the locomotor stimulatory effect of quinpirole was inhibited by both drugs. The present findings confirm that D_2 receptors play a primary role in the d-amphetamine discriminative cue, while the precise role of D_1 receptors remains to be disclosed.

d-Amphetamine discriminative cue; Dopamine D_1 receptors; Dopamine D_2 receptors; Receptor interactions; Dopamine receptor agonists; Dopamine receptor antagonists

1. Introduction

Compounds which selectively interact with D_1 or D_2 dopamine (DA) receptors have provided important information concerning the functional role of these distinct recognition sites (see Clark and White, 1987; Waddington and O'Boyle, 1989 for reviews). Evidence suggests that D_1 and D_2 receptors can interact in either a synergistic or in an opposing fashion. Of particular interest is the suggestion that a critical level of D_1 receptor stimulation is required for the expression of certain functional effects mediated by D_2 receptor stimulation. Behavioural work has provided three main pieces of evidence for this hypothesis. Firstly, selective D_2 receptor agonists cannot reverse the akinesia produced by the monoamine-depleting agent reserpine unless D_1 receptors are concurrently stimulated by D_1 receptor agonists (Gershanik et al., 1983; Jackson and Hashizume, 1986; White et al., 1988). Secondly, the locomotor stimulatory effects of D_2 agonists are inhibited by selective D_1 antagonists (Mailman et al., 1984; Pugh et al., 1985). Finally, the full spectrum of stereotypies produced by mixed D_1/D_2 agonists (e.g. apomorphine and d-amphetamine) is not observed with selective D_2 agonists unless they are administered with D_1 agonists (Barone et al., 1986; Arnt et al., 1987, 1988). A synergistic interaction between D_1 and D_2 receptors has also been observed in electrophysiological studies (Carlson et al., 1987; White, 1987; Wachtel et al., 1987).

Nielsen and colleagues (1989) have recently provided evidence that D_1 and D_2 receptors are both involved in the discriminative cue produced by the indirect DA agonist d-amphetamine. Antagonists selective for either D_1 or D_2 receptors blocked the cueing properties of d-amphetamine (see also Nielsen and Jepsen, 1985). On the other hand, selective D_2 agonists substituted for the stimulant while a variety of selective D_1 agonists did not generalise. Interestingly, the D_1 antagonist SCH 23390 did not inhibit pergolide substitution for the d-amphetamine cue despite blocking the motor activating effects of this D_2 agonist in a different group of rats. On the basis of these findings, Nielsen et al. (1989) concluded that D_2 receptors play a primary role in the d-amphetamine discriminative cue. In addition, these investigators suggested that D_1 and D_2 receptors in the dopaminergic system mediating d-amphetamine discrimination are less tightly coupled than those in dopaminergic motor systems.

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We have characterised the role of D_1 and D_2 receptors in d-amphetamine (0.5 mg/kg) discrimination by determining the ability of selective agonists to substitute for, and selective antagonists to inhibit, the cue. In addition, we have examined the ability of SCH 23390 and the selective D_2 antagonist YM 09151-2 to inhibit quinpirole substitution for d-amphetamine, and the locomotor stimulatory effects of the D_2 agonist. The latter experiment was carried out in the drug discrimination rats, to rule out the possibility that the differential effect of SCH 23390 observed by Nielsen and colleagues (1989) in these paradigms was not related in some way to the long-term repeated administration of d-amphetamine. Parts of this work have been reported in abstract form elsewhere (Furmidge et al., 1989a, b).

2. Materials and methods

2.1. Animals

Fifty six male, Sprague-Dawley albino rats (Harlan Olac Ltd., Bicester, U.K.) were food-deprived to approximately 85% of their free feeding weights (initial weight 250-300 g) by restricting food intake to an average of 20 g rat chow per day. Water was freely available. The rats were housed in pairs in a room maintained at a constant temperature (21-23°C). Lights were on from 07:00 to 19:00 h.

2.2. Apparatus

The drug discrimination experiments were conducted in four identical, commercially manufactured operant boxes (Campden Instuments Ltd., U.K.), each measuring 20 cm high with a 25×23 cm grid floor. Each box contained two retractable levers mounted on one wall, with a food hopper positioned equidistant between the levers. Food pellets were delivered by automatic dispensers. The boxes were dimly lit by a centrally positioned 2.8 W light and were enclosed in light- and sound-attenuating chambers. Fans provided ventilation and masking noise. Equipment was controlled, and data collected, by an Acorn Atom microcomputer (Acorn Computers Ltd., Cambridge, U.K.) using programs written in ONLIBASIC (Paul Fray Ltd., Cambridge, U.K.)

The activity experiment was conducted in four identical clear Perspex boxes measuring $35 \times 35 \times 30$ cm and containing a metal grid floor (1 cm^2 mesh). Screens were placed between the individual boxes to prevent visual distraction. Locomotor activity was measured by means of two infra-red photocells and associated detectors. The photocells were positioned on the sides of the box, 18 cm apart and 5.5 cm above the floor. The detectors were linked to an Acorn Atom microcomputer which was programmed in ONLIBASIC to record photocell interruptions of greater than 0.5 s duration.

2.3. Drug discrimination procedures

The discrimination procedures have been detailed elsewhere for a study using the same population of rats (Exner et al., 1989). Initially, rats were randomly assigned to one of the four operant boxes. For each box, half of the animals were randomly assigned to the right lever as the drug-appropriate lever and the other half to the left lever. This arrangement, and the fact that saline and drug sessions followed each other in a pseudorandom order, served to control for the development of position cues based on olfactory stimuli by animals that were run in the same box (Extance and Goudie, 1981).

Throughout the study, rats were injected i.p. with either 0.5 mg/kg d-amphetamine sulphate (the training drug), or 0.9% saline, 15 min before being placed in the operant boxes. Injection volumes were 1 ml/kg.

Only the correct lever was present during the initial phase of training. Animals were injected with saline and trained to press the saline-appropriate lever for food pellets. Following reliable performance under the saline condition, they learnt to respond on the drug-appropriate lever following d-amphetamine injections. Saline and d-amphetamine sessions were then carried out each day on a random basis with the restriction that neither condition occurred more than three days in a row. The rats were trained to respond on a fixed ratio (FR) 20 schedule on each lever using an ascending FR schedule independently for each condition.

Discrimination training was initiated once the rats were responding reliably on the FR20 schedule. During these training sessions of 25 min duration, both levers were available, but only responses on the appropriate lever were reinforced on an FR20 schedule. Incorrect responses were recorded but had no programmed consequences. The time in seconds to complete the first 20 responses on the correct lever (latency) and the percentage of drug lever responses prior to delivery of the first food pellet ($100 \times$ no. of drug lever responses divided by no. of drug + saline lever responses) were recorded. A response rate measure was derived by calculating the number of responses per minute before delivery of the first reinforcer ($60 \times$ no. responses before food pellet delivery divided by latency).

Discrimination training continued until rats reached criterion performance (nine out of ten consecutive sessions with more than 80% correct lever presses before the first reinforcement was delivered). Each animal was then randomly assigned to a subgroup of rats ($N = 8$, if not stated otherwise) for testing with a

specific drug or drug combination. No reinforcement was given during test sessions; the rat was removed from the operant box on completion of twenty responses on either of the levers. The number of drug- and saline-appropriate lever responses, and the latency to complete twenty responses on either of the levers were recorded. Each latency value was converted to a response rate measure.

Animals were randomly selected to participate in a series of generalisation and antagonism studies with compounds selective for either D_1 or D_2 receptors. They were injected with different doses of drug in a random order to determine whether these compounds substitute for d-amphetamine or block the cueing properties of this stimulant. The effects of different DA antagonists on quinpirole substitution for d-amphetamine was also examined. Animals received quinpirole (250 μg/kg) either alone, or in combination with SCH 23390 (50 μg/kg) or YM 09151-2 (50 μg/kg). Each rat received these drug conditions in a random order. If any animal failed to produce a minimum of 80% d-amphetamine-appropriate responses in the quinpirole control session, it was excluded from the study and another animal was used. Eight of thirteen rats reached this criterion and were included in the analyses.

Test sessions were separated by at least one d-amphetamine and one saline baseline session which provided control data. If discrimination performance deteriorated between test sessions, further training was carried out until performance restabilised. If under any condition an animal failed to respond with at least ten lever presses in a session, the data were considered unreliable and excluded from analysis. Similarly, data were not analysed if less than half of the rats tested under a condition failed to respond with at least 10 presses.

2.4. Locomotor activity procedures

The ability of YM 09151-2 (50 μg/kg) and SCH 23390 (50 μg/kg) to inhibit the locomotor hyperactivity produced by quinpirole was assessed. A group of the discrimination rats described above were used. These rats were maintained on a pseudo-random 0.5 mg/kg d-amphetamine/saline regime, except on days where activity testing took place.

Animals were habituated to the perspex photocell boxes for a period of 60 min. They were then injected with the relevant drugs, and locomotor activity was monitored for 1 h. Locomotion counts were recorded when the photocell beams were interrupted in strict sequence, i.e. the rat moved through the front beam and then the rear beam, or vice versa.

2.5. Statistical analyses

For each drug discrimination study, saline and d-amphetamine control values were obtained by averaging the control data for each of the relevant baseline sessions which preceded drug tests. In order to normalise distributions, drug lever responding percentages were arcsin transformed and response rates were logarithmically transformed. The effects of drugs on these measures were then analysed using the SAS-GLM procedure one-way analysis of variance for a repeated measures design (SAS Institute, Cary, NC, U.S.A.). Provided that this procedure yielded significant overall F values, comparisons between means were carried out using Duncan's test. Dose-dependent effects on discrimination were analysed using the SAS-PROBIT procedure in order to generate ED_{50} and ID_{50} values. Activity counts were square root transformed $[\sqrt{(x+0.5)}]$ and analysed by a one-way ANOVA.

2.6. Drugs

Unless stated otherwise, the following drugs were administered i.p. in 0.9% saline 30 min prior to testing: d-amphetamine sulphate (Sigma Chemical Co. Ltd., Poole, U.K.), 15 min prior; quinpirole HCl (Eli Lilly Co., Indianapolis, U.S.A.*); raclopride tartrate (Astra, Södertalje, Sweden*), s.c. 60 min prior; RU 24213 (N-n-propyl-n-phenylethyl-p-(3-hydroxyphenyl)ethylamine) (Roussel, France*); SCH 23390 (R-(+)-8-chloro-2,3,4,5,-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol maleate) (Schering, New Jersey, U.S.A.*); SKF 38393 (2,3,4,5,-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine HCl), in distilled water, and SKF 83566 (7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine HBr) (Smith Kline & French, Philadelphia, U.S.A.*); SKF 81297 (2,3,4,5-tetrahydro-6-chloro-7,8-dihydroxy-1-phenyl-1H-3-benzazebine HBr) (Lundbeck, Copenhagen, Denmark*) and YM 09151-2 (N(2RS,3RS)-1-benzyl-2-methyl-3-pyrrolidinyl-5-chloro-2-methoxy-4-methylaminobenzamide) (Yamanouchi Pharmaceuticals, Tokyo, Japan), in a minimum amount of 0.1 M acetic acid, neutralised with 0.1 M NaOH and brought up to volume with saline. All drugs were injected in a volume of 1 ml/kg except SKF 38393, which was injected in a volume of 2 ml/kg.

3. Results

3.1. Drug discrimination substitution studies

The selective D_2 agonists quinpirole and RU 24213 dose dependently increased drug lever responding (fig. 1), with the highest two doses of each drug producing

complete substitution (ED_{50}s = 91 and 250 μg/kg, respectively; see table 1 for statistical analysis). In contrast, the D_1 agonists SKF 38393 and SKF 81297 failed to substitute completely for d-amphetamine. While SKF 38393 always produced saline-appropriate responding, some doses of SKF 81297 produced responding which was significantly different from the saline condition. Thus, a weak partial substitution was obtained (fig. 1 and table 1).

All four DA agonists dose dependently reduced response rate (fig. 1 and table 1). This response disruption was particularly marked with the D_1 agonists. Three of the animals administered 16.0 mg/kg SKF 38393 failed to meet the minimum requirement of 10 lever presses, and the first five animals tested with 2.0 mg/kg SKF 81297 did not respond (data not shown). Observation of the behaviour of the animals during these sessions failed to reveal any obvious motor disruption.

3.2. Drug discrimination antagonism studies

The selective D_2 antagonist raclopride completely blocked the discriminative cue produced by d-amphetamine (ID_{50} = 420 μg/kg; fig. 2 and table 1). While YM 09151-2 also dose dependently reduced drug-appropriate responding, complete antagonism could not be demonstrated in a reliable (> 50%) number of rats. Only three animals completed the minimum requirement of 10 responses after administration

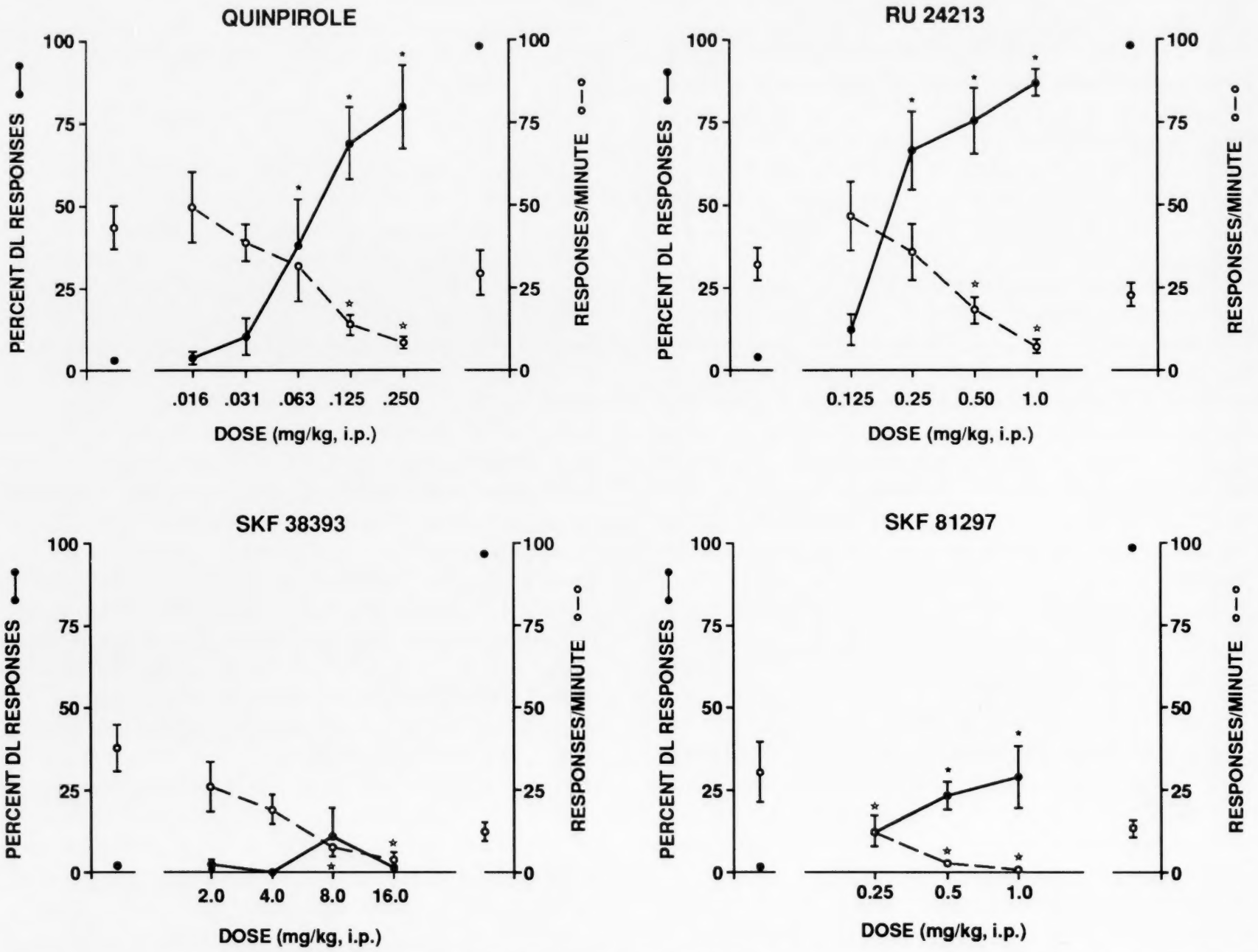

Fig. 1. Shows that the selective D_2 agonists quinpirole and RU 24213, but not the selective D_1 agonists SKF 38393 and SKF 81297, completely substitute for 0.5 mg/kg d-amphetamine. Filled circles represent the percentage of drug lever (DL) responses (±S.E.M.) in test sessions. Empty circles (±S.E.M.) represent the number of responses per minute at different drug doses. Asterisks indicate scores that differ significantly from saline performance ($P < 0.05$). Control performance (±S.E.M.) for saline is shown on the left, for d-amphetamine on the right side of the dose-response curve. These are the mean values of all sessions immediately preceding test sessions. All points represent the mean values of eight animals in the D_2 agonist studies. Six rats participated in the SKF 38393 study, but only five met the minimum requirement of 10 lever presses after 8.0 mg/kg and three after 16.0 mg/kg. Eight rats participated in the SKF 81297 study; seven, five and five rats responded after the three doses shown. All five animals administered 2.0 mg/kg failed to press the lever on more than 10 occasions.

of 100 μg/kg, although each of these responded almost entirely on the saline lever (data not shown). The estimated ID_{50} value for YM 09151-2 was 14 μg/kg. Raclopride also produced a marked reduction in response rate and, like YM 09151-2, a significant rate-reducing effect was observed at a lower dose than that required to attenuate d-amphetamine discrimination. Only four animals responded more than 10 times after the highest dose of raclopride (fig. 2 and table 1). Animals receiving high doses of either D_2 antagonist remained still for most of the experimental session and displayed some form of muscular rigidity.

SCH 23390 and SKF 83566 also completely antagonised d-amphetamine-appropriate responding (ID_{50}s = 13 and 63 μg/kg, respectively; fig 2. and table 1). Notably, SCH 23390 did not reduce response rate below that observed in d-amphetamine control sessions. Although higher doses of SKF 83566 did inhibit response rate, the effects of this drug were less marked than observed with D_2 antagonists.

Since SKF 38393 is known to be a partial agonist at D_1 receptors, we considered whether it could reduce the cueing properties of d-amphetamine. The levels of drug lever responding after the combination of d-amphetamine and 4.0-8.0 mg/kg SKF 38393 were not different to that observed in d-amphetamine control sessions (d-amp control: 99.2 + 0.5%, n = 6; d-amp + 4.0 mg/kg SKF 38393: 77.3 + 19.7%, n = 4; d-amp + 8.0 mg/kg SKF 38393: 100.0%, n = 3). These doses of the partial D_1 agonist significantly reduced response rate and some rats did not meet the minimum requirement of 10 lever presses (data not shown). Only two of

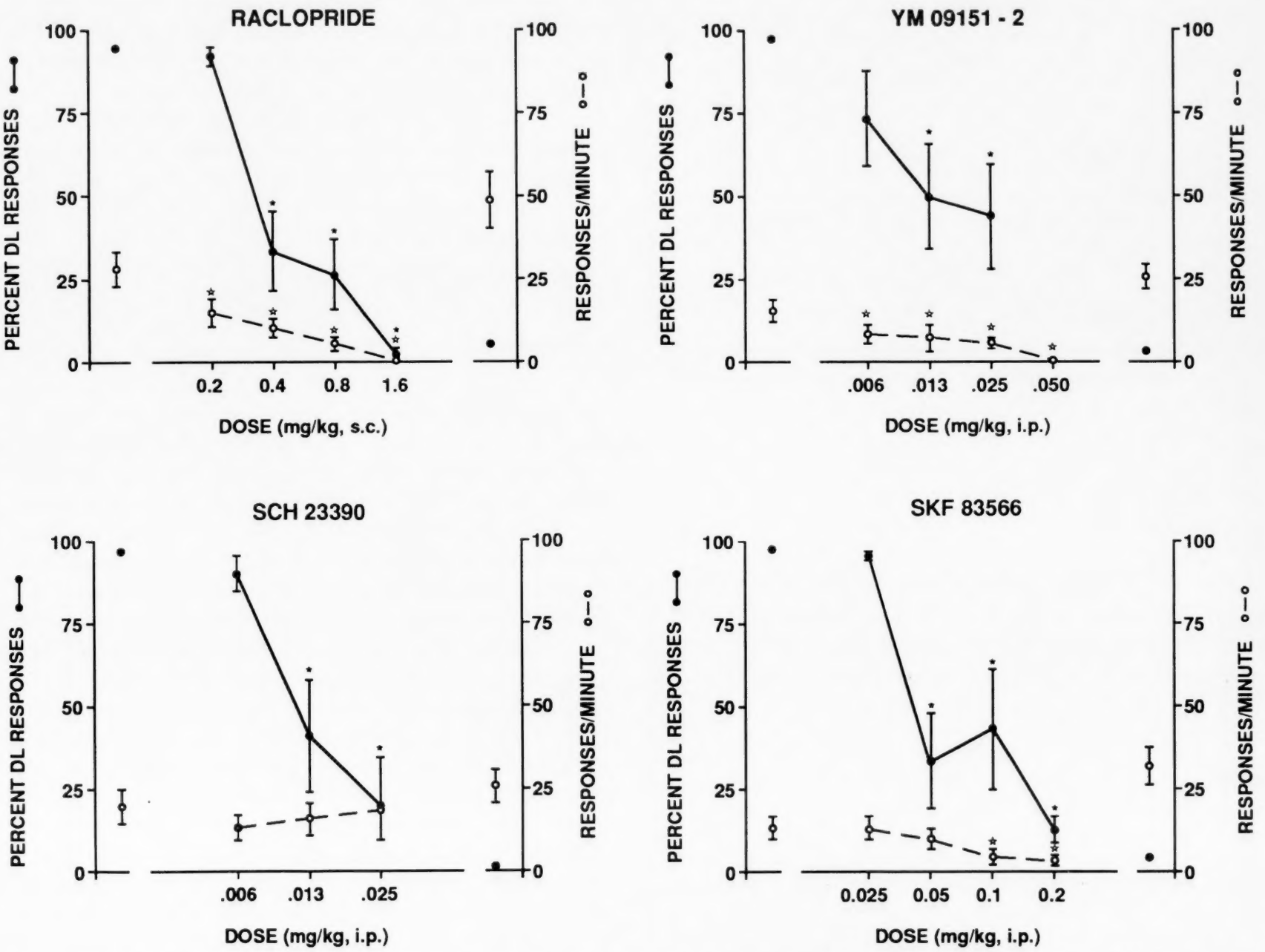

Fig. 2. Illustrates the dose-dependent inhibition of d-amphetamine discrimination by the selective D_2 agonists raclopride and YM 09151-2, and selective D_1 antagonists SCH 23390 and SKF 38566 (N = 8 per study). Filled circles represent the percentage of drug lever responses (±S.E.M.) after different doses of DA antagonist in combination with 0.5 mg/kg d-amphetamine. Empty circles (±S.E.M.) represent responses per minute. Control performance (±S.E.M.) for d-amphetamine is shown on the left of the figure, and for saline on the right. Asterisks represent scores which differed significantly from d-amphetamine responding. Only four rats completed 10 responses after 1.6 mg/kg raclopride. The number of rats meeting this minimum requirement after each dose of YM 09151-2 were seven, six, five and three, respectively, and after SKF 83566 seven, seven, six and six.

TABLE 1

ANOVA tables for the drug discrimination studies. Shown are the F values and degrees of freedom (top line) and significance levels (bottom line) for each drug. n.s. is equivalent to $P > 0.05$

Drug	Discrimination	Response rate
Agonist		
Quinpirole	$F(6,40) = 18.15$	$F(6,42) = 11.20$
	$P < 0.0001$	$P < 0.0001$
RU 24213	$F(5,35) = 21.54$	$F(5,35) = 14.63$
	$P < 0.0001$	$P < 0.0001$
SKF 38393 [a]	$F(5,21) = 177.22$	$F(5,24) = 12.14$
	$P < 0.0001$	$P < 0.0001$
SKF 81297	$F(4,24) = 122.81$	$F(4,28) = 23.14$
	$P < 0.0001$	$P < 0.0001$
Antagonist		
Raclopride	$F(5,31) = 25.75$	$F(5,35) = 17.32$
	$P < 0.0001$	$P < 0.0001$
YM 09151-2	$F(4,22) = 7.35$	$F(5,35) = 18.92$
	$P < 0.02$	$P < 0.0001$
SCH 23390	$F(4,25) = 17.79$	$F(4,28) = 2.48$
	$P < 0.0001$	n.s
SKF 83566	$F(5,28) = 19.35$	$F(5,35) = 16.14$
	$P < 0.0001$	$P < 0.0001$
SKF 38393 [a]	$F(3,10) = 22.16$	$F(4,20) = 7.62$
	$P < 0.0001$	$P < 0.0007$

[a] The multiple comparison procedures revealed that the effects of all drug doses were not different to relevant control values. The significant F values reflect differences between the d-amphetamine and vehicle control groups.

the rats responded when 16.0 mg/kg SKF 38393 was administered in conjunction with d-amphetamine, and these did so on the drug lever. No obvious signs of motor disturbance were noted.

3.3. Antagonism of quinpirole substitution for the d-amphetamine cue

YM 09151-2 (50 μg/kg) completely blocked the ability of quinpirole (250 μg/kg) to substitute for d-amphetamine. SCH 23390 (50 μg/kg) exerted only weak attenuating effects. Both drug combinations strongly reduced response rate, and a number of rats failed to meet the minimal response requirements (table 2).

TABLE 2

Ability of YM 09151-2 (50 μg/kg) and SCH 23390 (50 μg/kg) to reduce the ability of quinpirole (250 μ/kg) to substitute for d-amphetamine (0.5 mg/kg). Effects on percent drug lever (DL) responses and on response rate are shown. Subject numbers are shown in parentheses

Drug	%DL responses	Responses/min
Saline control	5.8 ± 1.3 (8)	21.5 ± 4.2 (8)
d-Amphetamine control	98.5 ± 0.5 (8)	19.2 ± 4.7 (8)
Quinpirole control	93.3 ± 1.7 (8)	9.8 ± 1.2 (8)
Quinpirole + YM 09151-2	6.6 ± 2.8 (5)	7.1 ± 3.3 (8)
Quinpirole + SCH 23390	77.8 ± 7.6 (5)	2.8 ± 1.8 (8)

3.4. Antagonism of quinpirole-induced locomotor hyperactivity

The increase in photocell counts produced by quinpirole (250 μg/kg) was blocked by 50 μg/kg YM 09151-2 (vehicle control: 7.0 ± 3.2; quinpirole control: 80.6 ± 25.9; YM 09151-2 + quinpirole: 2.8 ± 1.2; $F(2,11) = 13.07$, $P < 0.002$), but not by 50 μg/kg SCH 23390 (data not shown). However, direct observation revealed that the rats treated with SCH 23390 and quinpirole did not exhibit any coordinated motor activity. A marked myoclonic jerking behaviour accounted for the photocell counts. The animals would remain immobile for a period of time and then spring into the air, sometimes to the 30 cm height of the activity box. This behaviour, observed in all five animals tested, was not quantified and the experiment was terminated. Jerking was not observed when these animals were used in the drug discrimination experiment with the same drug combination, or when YM 09151-2 was administered with quinpirole.

4. Discussion

Little question arises concerning the importance of D_2 DA receptors in mediating the discriminative effects of d-amphetamine. The present studies with quinpirole and RU 24213 confirm earlier reports that selective D_2 agonists completely substitute for d-amphetamine (Arnt, 1988; Nielsen et al., 1989). Moreover, the complete antagonism of d-amphetamine discrimination by raclopride and YM 09151-2 is consistent with previous findings with raclopride and other selective D_2 antagonists (Nielsen and Jepsen, 1985; Arnt, 1988; Nielsen et al., 1989).

The role of D_1 DA receptors in the d-amphetamine discriminative cue is less clear. Both SCH 23390 and SKF 83566 potently blocked d-amphetamine discrimination. The findings with SCH 23390 confirm earlier reports (Nielsen and Jepsen, 1985; Arnt, 1988; Exner et al., 1989; Nielsen et al., 1989). In contrast to the effects observed with D_2 agonists, the selective D_1 agonists SKF 38393 and SKF 81297 failed to completely substitute for d-amphetamine. Similar findings have been previously observed with these compounds and other derivatives such as SKF 89626 and SKF 75670 in animals trained to discriminate d-amphetamine from saline (Arnt, 1988; Nielsen et al., 1989).

These findings indicate that both D_1 and D_2 recep-

tors are involved in the d-amphetamine discriminative cue although their precise functional role seems to be different. Stimulation of D_2 receptors appears to play a primary role, while activation of D_1 receptors may be a necessary but not sufficient condition for d-amphetamine discrimination. This idea is consistent with the recent hypothesis concerning an 'enabling' role for D_1 receptors in the functional expression of certain D_2 receptor-mediated behavioural effects (Clark and White, 1987; Waddington and O'Boyle, 1989 for reviews). However, a number of factors must be considered before the present findings can be interpreted in this manner.

It might be argued that the failure of SKF 38393 and SKF 75670 to generalise to d-amphetamine is due to the partial agonist activity of these compounds at D_1 receptors regulating adenylate cyclase activity (Setler et al., 1978; Arnt et al., 1988; Nielsen et al., 1989). Such a pharmacological profile has been suggested by certain behavioural studies; for example, high doses of both compounds partially inhibit methylphenidate-induced gnawing in mice (Nielsen et al., 1989). However, in the present study SKF 38393 not only failed to mimic the d-amphetamine cue but also did not reduce discriminability of the drug. In contrast, partial D_2 DA agonists can reduce, and partially substitute for, the d-amphetamine discriminative cue (Exner et al., 1989; Furmidge, 1990). Nielsen and colleagues (1989) observed very minor inhibitory effects (approximately 15%) with SKF 38393 and some inhibition (approximately 30%) with one of two tested doses of SKF 75670. These investigators stated that the partial agonist action of these compounds alone cannot explain their failure to substitute for d-amphetamine, since the full D_1 agonist SKF 81297 also failed to completely generalise to d-amphetamine. However, Arnt et al. (1987), in contrast to Nielsen and colleagues, have reported that SKF 81297 also acts as a partial agonist at D_1 receptors regulating adenylate cyclase activity, exhibiting similar intrinsic activity to SKF 38393. Moreover, Arnt and colleagues (1988) has expressed reservations in relating the intrinsic activity of D_1 agonists determined in the adenylate cyclase model to their behavioural effects.

A precise understanding of the role of D_1 receptors in d-amphetamine discrimination is further complicated by other findings. Although SKF 81297 failed to completely substitute for d-amphetamine, it did significantly increase drug lever responding (see also Nielsen et al., 1989). In both Nielsen's and the present study, the effects of higher doses of drug could not be examined due to the marked response disruption. However, the level of drug lever responding did appear to asymptote rather than show a reasonable dose dependency. The relevance of this observation is unclear. It is possible that D_1 agonists produce more subtle stimulus effects than those produced by a large increase in DA release following d-amphetamine (0.5-1.0 mg/kg) administration. We have examined whether SKF 81296 can generalise to a lower dose of d-amphetamine by retraining some of our animals to discriminate 0.125 mg/kg d-amphetamine from saline. All three of the animals which learnt this task responded almost entirely on the drug lever after 0.5 mg/kg SKF 81297 (Furmidge, 1990). This finding would suggest that the low level of drug lever responding observed after SKF 81297 in the 0.5 mg/kg d-amphetamine study was not the result of random responding. However, these preliminary findings should be interpreted cautiously since a significant peripheral component may underlie the stimulus properties of low doses of d-amphetamine (Colpaert et al., 1976; Stolerman and D'Mello, 1981). This problem can be circumvented by determining whether centrally administered D_1 agonists can substitute for low doses of d-amphetamine. It should be noted that partial substitution of about 50% has been observed with centrally injected fenoldopam, SKF 38393 and SKF 75670 in animals trained to discriminate 1.0 mg/kg d-amphetamine from saline (unpublished findings quoted in Nielsen and Scheel-Krüger, 1988). Clearly, all these findings emphasise that the precise role of D_1 receptors in d-amphetamine discrimination is not yet known.

The ability of quinpirole to substitute for d-amphetamine was blocked by the selective D_2 antagonist YM 09151-2. However, while SCH 23390 completely blocked the cueing properties of d-amphetamine, a higher dose of this D_1 antagonist only minimally influenced quinpirole substitution. Nielsen and colleagues (1989) reported no inhibition of pergolide substitution for d-amphetamine by SCH 23390. The failure of SCH 23390 to block D_2 agonist substitution for d-amphetamine contrasts with the inhibitory effect of this drug on the locomotor stimulation produced by the same compounds in normal rats (Mailman et al., 1984; Pugh et al., 1985; Nielsen et al., 1989) and in animals which have received long-term periodic d-amphetamine (present findings). Thus, there appear to be differences in the dopaminergic mechanisms which underlie the motor stimulatory effects of D_2 agonists and their cueing properties. This idea is supported by other findings. Dopamine D_1 agonists do not potentiate the ability of D_2 agonists to generalise to d-amphetamine, despite enhancing their unconditioned behavioural effects (Nielsen et al., 1989; Furmidge, 1990). Moreover, the discriminative effects of a postsynaptic activating dose of the D_2 agonist (−)-NPA (in animals trained to discriminate this drug from saline) are not blocked by D_1 antagonists (Arnt, 1988). In light of these findings, Nielsen and colleagues (1989) suggested that dopaminergic motor systems may have a tight coupling between D_1 and D_2 receptors, 'whereas

the DA systems involved in d-amphetamine discrimination could allow DA-like effects by D-2 receptor stimulation with a minimum of D-1 tone'. Although the locomotor stimulation and discriminative effects of d-amphetamine are thought to be mediated by enhanced DA function in the nucleus accumbens (Kelly et al., 1975; Costall et al., 1977; Nielsen and Scheel-Krüger, 1986), they do not appear to have a common neural substrate.

It is puzzling that blockade of D_1 receptors influences d-amphetamine, but not quinpirole or pergolide, substitution in animals trained to discriminate d-amphetamine from saline. Nielsen and Scheel-Krüger (1988) speculate, 'that at some point in the chain of synapses involved in CNS-stimulant cues, D-1 receptors may be preceding D-2 receptors'. We interpret this hypothesis in the following way: release of DA by d-amphetamine may only influence the population of D_2 receptors critical for the perception of the cue following activation of 'upstream' D_1 receptors. Blockade of D_1 receptors would preclude perception of d-amphetamine since no D_2 receptor stimulation would occur. On the other hand, the effects of selective D_2 agonists would not be influenced, since D_1 receptor blockade would not influence direct stimulation of 'downstream' D_2 receptor sites. However, although this is an interesting attempt to account for the drug discrimination data, there is at present no anatomical evidence supporting this form of neural arrangement (see Furmidge, 1990 for further discussion).

Although we observed that the locomotor stimulatory effect of quinpirole in our discrimination rats was blocked by SCH 23390, the resulting behaviour was clearly not normal. Animals exhibited strong myoclonic jerks during the time that they were otherwise immobile. This behaviour was not reported by Nielsen and colleagues (1989), who used drug naive rats, or by various other investigators and, therefore, may be related to the fact that our animals had been receiving long-term periodic injections of d-amphetamine. However, Waddington and colleagues have observed myoclonic jerks in normal animals receiving a combination of D_1 antagonist (SCH 23390 or R-SKF 83566) and selective D_2 agonist (RU 24213 and LY 163502) (Waddington et al., 1986, 1988; see also Grabowska-Andén and Andén, 1983, 1987). Strangely, myoclonic jerks were not observed during the drug discrimination study with SCH 23390 and quinpirole. In this case, animals were injected with the drugs and remained in their home cage (with one other rat) before being placed in the operant chamber. During the activity study, the rats were injected with the drugs and replaced into the open-topped activity boxes. It is therefore possible that an environmental influence plays a part in the expression of this behaviour, a point which merits investigation.

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References

Arnt, J., 1988, The discriminative stimulus properties of the D-1 agonist SK&F 38393 and the D-2 agonist (−)-NPA are mediated by separate mechanisms, Life Sci. 42, 565.

Arnt, J., J. Hyttel and E. Meier, 1988, Inactivation of dopamine D-1 or D-2 receptors differentially inhibits stereotypies induced by dopamine agonists in rats, Eur. J. Pharmacol. 155, 37.

Arnt, J., J. Hyttel and J. Perregaard, 1987, Dopamine D-1 receptor agonists combined with the D-2 agonist quinpirole facilitate the expression of oral stereotyped behaviour in rats, Eur. J. Pharmacol. 133, 137.

Barone, P., T.A. Davis, A.R. Braun and T.N. Chase, 1986, Dopaminergic mechanisms and motor function: Characterization of D-1 and D-2 dopamine receptor interactions, Eur. J. Pharmacol. 123, 109.

Carlson, J.H., D.A. Bergstrom and J.R. Walters, 1987, Stimulation of both D_1 and D_2 dopamine receptors appears necessary for full expression of postsynaptic effects of dopamine agonists: a neurophysiological study, Brain Res. 400, 205.

Clark, D. and F.J. White, 1987, Review: D1 dopamine receptor – The search for a function: A critical evaluation of the D1/D2 dopamine receptor classification and it's functional implications, Synapse 1, 347.

Colpaert, F.C., J.J.M.D. Kuyps, C.J.E. Niemegeers and P.A. Janssen, 1976, Discriminative stimulus properties of a low dl-amphetamine dose, Arch. Int. Pharmacodyn. 223, 34.

Costall, B., C.D. Marsden, R.J. Naylor and C.J. Pycock, 1977, Stereotyped behaviour patterns and hyperactivity induced by amphetamine and apomorphine after discrete 6-hydroxydopamine lesions of extrapyramidal and mesolimbic nuclei, Brain Res. 123, 89.

Exner, M., L.J. Furmidge and D. Clark, 1989, Inhibitory effects of partial D2 dopamine receptor agonists on the d-amphetamine discriminative cue, Behav. Pharmacol. 1, 101.

Extance, K. and A.J. Goudie, 1981, Inter-animal olfactory cues in operant drug discrimination procedures in rats, Psychopharmacolgy 73, 363.

Furmidge, L.J., 1990, Effects of Partial Dopamine D2 Receptor Agonists on d-Amphetamine-induced Behaviour (Ph.D. Thesis, University of Reading, U.K.).

Furmidge, L.J., M. Exner and D. Clark, 1989a, Involvement of D1 and D2 dopamine receptors in the d-amphetamine discriminative cue, J. Psychopharmacol. 3, 1P.

Furmidge, L.J., M. Exner and D. Clark, 1989b, The d-amphetamine discriminative cue: Involvement of D1 and D2 dopamine receptors, Behav. Pharmacol. 1 (Suppl. 1), 10.

Gershanik, O., R.E. Heikkila and R.C. Duvoisin, 1983, Behavioral correlations of dopamine receptor activation, Neurology 33, 1489.

Grabowska-Andén, M. and N.E. Andén, 1983, Stimulation of postsy-

naptic DA-2 receptors produces jerks in reserpine-treated rats, J. Pharm. Pharmacol. 35, 543.

Grabowska-Andén, M. and N.E. Andén, 1987, Inhibitory role of D-1 dopamine receptors for the jerks induced by BHT 920 in rats, J. Pharm. Pharmacol. 39, 660.

Jackson, D.M. and M. Hashizume, 1986, Bromocriptine induces marked locomotor stimulation in dopamine-depleted mice when D-1 dopamine receptors are stimulated with SKF 38393, Psychopharmacology 90, 147.

Kelly, P.H., P.W. Seviour and S.D. Iversen, 1975, Amphetamine and apomorphine responses in the rat following 6-OHDA lesions of the nucleus accumbens septi and corpus striatum, Brain Res. 94, 507.

Mailman, R.B., D.W. Schultz, M.H. Lewis, L. Staples, H. Rollema and D.L. Dehaven, 1984, SCH 23390: A selective D1 dopamine antagonist with potent D_2 behavioural actions, Eur. J. Pharmacol. 101, 159.

Nielsen, E.B. and S.A. Jepsen, 1985, Antagonism of the amphetamine cue by both classical and atypical antipsychotic drugs, Eur. J. Pharmacol. 111, 167.

Nielsen, E.B., K. Randrup and P.H. Andersen, 1989, Amphetamine discrimination: Effects of dopamine receptor agonists, Eur. J. Pharmacol. 160, 253.

Nielsen, E.B. and J. Scheel-Krüger, 1986, Cueing effects of amphetamine and LSD: Elicitation by direct microinjection of the drugs into the nucleus accumbens, Eur. J. Pharmacol. 125, 85.

Nielsen, E.B. and J. Scheel-Krüger, 1988, CNS stimulants: Neuropharmacological mechanisms, in: Transduction Mechanisms of Drug Stimuli, eds. F. C. Colpaert and R. L. Balster (Springer-Verlag, Berlin) p. 57.

Pugh, M.T., K.M. O'Boyle, A.G. Molloy and J.L. Waddington, 1985, Effects of the putative D-1 antagonist SCH 23390 on stereotyped behaviour induced by the D-2 agonist RU 24213, Psychopharmacology 87, 308.

Setler, P.E., H.M. Sarau, C.L. Zirkle and H.L. Saunders, 1978, The central effects of a novel dopamine agonist, Eur. J. Pharmacol. 50, 419.

Stolerman, I.P. and G.D. D'Mello, 1981, Role of training conditions in discrimination of central nervous system stimulants by rats, Psychopharmacology 73, 295.

Wachtel, S.R, X.-T. Hu, M.P. Galloway and F.J. White, 1987, D1 dopamine receptor stimulation enables the postsynaptic, but not autoreceptor, effects of D2 agonists in nigrostriatal and mesoaccumbens dopamine systems, Synapse 4, 327.

Waddington, J.L., A.G. Molloy, K.M. O'Boyle and M. Mashurano, 1986, Motor consequences of D1-dopamine receptor stimulation and blockade, Clin. Neuropharmacol. 9, 20.

Waddington, J.L., A.M. Murray and K.M. O'Boyle, 1988, New selective D-1 and D-2 dopamine receptor agonists as further probes for behavioural interactions between D-1 and D-2 system, in: Pharmacology and Functional Regulation of Dopaminergic Neurons, eds. P. Beart, G. Woodruff and D. Jackson (MacMillan Press, London) p. 63.

Waddington, J.L. and K.M. O'Boyle, 1989, Drugs acting on brain dopamine receptors: A conceptual re-evaluation five years after the first selective D-1 antagonist, Pharmacol. Ther. 43, 1.

White, F.J., 1987, D-1 dopamine receptor stimulation enables the inhibition of nucleus accumbens neurons by a D-2 receptor agonist, Eur. J. Pharmacol. 135, 101.

White, F.J., L. Bednarz, S.R. Wachtel, S. Hjorth and R. Brooderson, 1988, Is stimulation of both D1 and D2 receptors necessary for the expression of dopamine-mediated behaviours, Pharmacol. Biochem. Behav. 30, 189.

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Segment-specific effects of the heat-stable enterotoxin of *E. coli* on electrolyte transport in the rat colon

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The heat-stable enterotoxin of *E. coli* (STa) induced an increase in short-circuit current (Isc) in the rat colon. The maximal increase in Isc was about three times larger in the proximal than the distal colon. The action of STa was mimicked by 8-Br-cyclic GMP. Unidirectional flux measurements revealed that STa decreased Na^+ and Cl^- absorption in the distal colon, while it decreased Na^+ absorption and activated Cl^- secretion in the proximal colon. In the distal, but not in the proximal colon, indomethacin inhibited the action of STa and of 8-Br-cyclic GMP. Inhibition by indomethacin could be overcome by addition of prostaglandin E_2 or forskolin, but not by addition of a non-hydrolysable analogue of cyclic AMP, suggesting an action of STa on cyclic AMP hydrolysis. Amrinone and trequinsin, two inhibitors of cyclic GMP-inhibited phosphodiesterases, mimicked the action of STa on Isc and inhibited the response to a subsequent administration of the toxin indicating the modulation of a cyclic GMP-inhibited phosphodiesterase by STa in the distal colon. The results give evidence for different intracellular action sites of STa in the two parts of the rat colon.

Enterotoxin of *E. coli* (heat-stable); Colon (rat); Ion transport; Prostaglandins; cGMP-inhibited phosphodiesterase

1. Introduction

The heat-stable enterotoxin of *E. coli* (STa), which is produced by some pathogenic strains of this bacterium, constitutes a family of low molecular weight peptides from 18 to 47 amino acid residues (for references see Giannella et al., 1983). STa of *E. coli* evokes a secretory diarrhea (for review see Giannella, 1981). Until now, the mechanism of action of this toxin has been studied almost exclusively in mammalian small intestine, where STa inhibits electrolyte absorption and stimulates secretion (Field et al., 1978; Rao and Field, 1984).

STa binds to specific sites in the intestinal membrane and activates the particulate guanylate cyclase (Guerrant et al., 1980; Rao et al., 1980). The consequence is an increase in the intracellular concentration of cyclic 3′,5′-guanosine monophosphate (cyclic GMP), which is finally responsible for the induction of secretion (Hughes at al., 1978).

The coupling between the STa receptor and guanylate cyclase is the object of controversy in the literature (for review see Waldman and Murad, 1987). A role has been attributed to arachidonic acid metabolism, because the cyclooxygenase blocker, indomethacin, inhibits the secretory response to STa in mouse intestine (Madsen and Knoop, 1978), and inhibits the activation of guanylate cyclase in rat and rabbit small intestine (Greenberg et al., 1980; Guerrant et al., 1980). A model has been proposed in which the STa receptor is coupled to guanylate cyclase by arachidonic acid metabolites (De Jonge, 1984). However, more recent data argue against a role of arachidonic acid metabolites in the mechanism of action of STa. In fact, STa does not alter the pattern of cellular lipids (Dreyfus et al., 1984) and also does not activate phospholipase A_2 (Waldman et al., 1986).

Besides the role for cyclic GMP, an involvement of Ca^{2+} in the mechanism of action of STa has been proposed, since antagonists of the Ca^{2+} pathway, e.g. lanthanum ions or chlorpromazine, have been shown to inhibit the STa response (Abbey and Knoop, 1979; Greenberg et al., 1980, 1982).

The present study was carried out to investigate the action of STa on the colon. Because ionic transport is not homogeneous throughout the colon (see e.g. Foster et al., 1986; Nobles et al., 1991), STa action was studied in the distal and the proximal part of the colon. All experiments were carried out in the presence of the

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neurotoxin, tetrodotoxin (TTX), in order to exclude neuronal actions of STa as described for the small intestine (Eklund et al., 1985).

2. Materials and methods

2.1. Solutions

Most of the experiments were carried out in a bathing solution containing (mM): NaCl 107, KCl 4.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, $CaCl_2$ 1.25, $MgSO_4$ 1 and glucose 12. The solution was gassed with carbogen (5% CO_2, 95% O_2) and kept at a temperature of 37 °C; the pH was 7.4. For the Cl^--free buffer NaCl was replaced with Na gluconate. The bicarbonate-free solution consisted of (mM): HEPES (N-(2-hydroxy-ethyl)piperazine-N′-2-ethansulfonic acid) 10, NaCl 140, KCl 4.5, $CaCl_2$ 1.25, $MgSO_4$ 1.0 and glucose 12. The pH was adjusted at 7.4 with NaOH, and the solution was gassed with O_2.

2.2. Tissue preparation

Female Wistar rats (180-200 g) were used. The rats had free access to water and food until the day of the experiment. The animals were killed by cervical dislocation. The tissue was prepared as previously described (Andres et al., 1985). Briefly, the serosa and the muscularis propria were stripped away by hand to obtain the mucosa-submucosa preparation of the colon. The colon (without the caecum) was divided in five segments of around 4 cm length. The segments were numbered continuously from 1 (the most distal segment, close to the rectum) to 5 (the most proximal segment, close to the caecum). Most experiments were carried out with segments 2 and 5 prepared from the same rat.

2.3. Short-circuit current measurement

The tissue was fixed in a modified Ussing chamber (Andres et al., 1985), bathed with a volume of 4 ml on each side of the mucosa and short-circuited by a voltage-clamp (Van Driessche and Lindemann, 1978). Subepithelial resistance was corrected for according to the method of Gebhardt (1979). The exposed surface of the tissue was 1 cm^2. Short-circuit current (Isc) was continuously recorded and tissue conductance (Gt) was measured every minute. The open-circuit potential difference (Pd) was calculated from these values.

2.4. Unidirectional flux measurements

Unidirectional transmural sodium and chloride fluxes were performed under short-circuit conditions using ^{22}Na and ^{36}Cl as tracers. After an equilibration period of 20 min, ^{22}Na (59 kBq) and ^{36}Cl (29 kBq) were added on one side of the epithelium. Two parallel chambers were employed under each set of conditions, one for the measurement of the mucosal to serosal flux (J_{ms}), and one for the measurement of the serosal to mucosal flux (J_{sm}). After an additional 20 min unidirectional fluxes were measured in four sequential 20-min periods. These periods were: control, TTX (1.25 μM serosal), STa (mucosal), and indomethacin (1 μM mucosal and serosal). Fifteen to twenty minutes were allowed after addition of TTX, indomethacin or STa, before the fluxes were measured for 20 min. The residual ion flux (J^R_{net}), i.e. the net transport of all ions other than Na^+ and Cl^-, was calculated from the difference between the arithmetic mean of the Na^+ and Cl^- net fluxes and the mean short-circuit current: $J^R_{net} = Isc - (J^{Na}_{net} - J^{Cl}_{net})$.

2.5. Experimental design

The efficiency of the commercially available STa to induce colonic secretion showed remarkable variation. The same batch of STa was therefore used in each series of experiments in order to avoid discrepancies in the action of the toxin. Pilot experiments were carried out in each series of experiments in order to define the concentrations which would evoke an about equieffective increase in Isc in the proximal and the distal colon.

After an equilibrium period of 20-30 min, STa was administered on the mucosal side. The response to STa was determined in the presence of TTX in order to exclude an action of STa by nervous mediation (Eklund et al., 1985). When Isc had stabilized, STa was washed out three times with 5-fold the chamber volume for 20-30 min. If the serosal compartment had to be washed out, TTX was added again at the end of the washing procedure. The putative antagonist was then added. STa was tested again after an equilibration period during which the Isc stabilized.

2.6. Data evaluation

The basal short-circuit current, just before addition of a substance, was determined as a mean over 5 min. The values are given as differences from the former baseline (ΔIsc). The plateau was measured as a mean over 5 min. In some cases, as indicated in the text, the maximal increase in Isc (peak) was measured.

2.7. Drugs

The methanol soluble fraction of the heat-stable enterotoxin of *E. coli* (STa) was added from an aqueous stock containing 0.1% bovine serum albumin (w/v). 8-Bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-

cyclic GMP), carbachol, 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate (CPT-cyclic AMP), 3-isobutyl-1-methylxanthine (IBMX), prostaglandin E_2 (PGE_2), trequinsin (gift from Hoechst, Frankfurt, FRG), 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8), tetrodotoxin (TTX), and verapamil were added from aqueous solutions. Amiloride, because of its poor solubility, was added by exchanging the mucosal solution with amiloride-containing buffer. Amrinone (gift from Sterling-Winthrop, Guildford Surrey, USA) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; gift from Prof. R. Greger, Freiburg, FRG) were dissolved in DMSO (final maximal concentration 0.25%, v/v). Forskolin, furosemide (Hoechst, Frankfurt, FRG), indomethacin (Sharp and Dohme, München, FRG) and trifluoperazine were added from ethanolic stock solutions (final maximum concentration 0.25%, v/v). If not indicated differently, drugs were from Sigma (Deisenhofen, FRG). Radioisotopes were from NEN (Dreieich, FRG); specific activities were 3.9 TBq/g for ^{22}Na and 550 MBq/g for ^{36}Cl.

2.8. Statistics

The result are given as means $\pm$ 1 S.E. The significance of differences was tested by analysis of variance and, if indicated, by paired or unpaired two-tailed Student's t-test or a U-test, respectively. An F-test was applied to decide which test method was to be used. $P < 0.05$ was considered to be significant. S.E.s for calculated values, i.e. net ion transport, were calculated according to the law of error propagation. Homogeneity of groups was tested by multifactor analysis of variance and the Scheffé test for comparison of linear contrasts.

3. Results

3.1. Action of STa on the electrical parameters of the rat colon

All experiments were performed with mucosal tissues pretreated with 1.25 μM TTX (added to the serosal side) in order to exclude neuronal actions of STa. The enterotoxin, administered to the mucosal side, induced an increase in Isc. Pilot experiments revealed that the response to STa was not homogeneous throughout the colon. In order to study the segment-dependent response to STa in more detail, the entire colon was divided into five segments. The heat-stable enterotoxin increased Isc in all segments in a concentration-dependent manner (fig. 1). The maximal Isc response evoked by STa increased from distal to proximal. The maximal increase in Isc was 70-85 $\mu A \cdot cm^{-2}$ in the proximal segments (3-5) and only

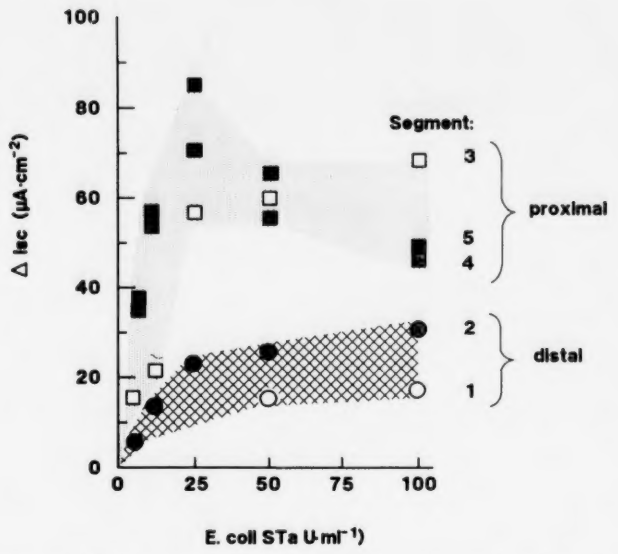

Fig. 1. *E. coli* STa concentration-response curves in the rat colon. The colon was divided in five segments of equal length, segment 1 adjoined the rectum, segment 5 adjoined the caecum. STa was applied on the mucosal side of tissues pretreated with TTX (1.25 μM at the serosal side). The values are means of Isc measured at the plateau in Isc induced by STa, n = 4-9. S.E. values were omitted for the sake of clarity.

15-25 $\mu A \cdot cm^{-2}$ in the distal segments (1-2). A multifactor analysis of variances (Scheffé test) revealed that segments 1 and 2 belonged to one homogeneous group and segments 3-5 belonged to another group.

Subsequent experiments were carried out with segment 2 as representative of distal segments and with segment 5 as representative of proximal segments. The concentrations of the toxin were adapted for each lot of STa to induce an equieffective increase in Isc in both segments.

Baseline electrical parameters were different in the proximal and the distal colon. The distal colon (in the presence of TTX) had a basal Isc of 44.1 ± 2.8 $\mu A \cdot cm^{-2}$, a Gt of 13.8 ± 0.5 $mS \cdot cm^{-2}$, and a Pd of 3.4 ± 0.2 mV (serosal side positive; n = 141). The baseline parameters for proximal colon were: Isc 51.9 ± 3.1 $\mu A \cdot cm^{-2}$, Gt 36.3 ± 1.5 $mS \cdot cm^{-2}$, and Pd 1.7 ± 0.1 mV (serosal side positive; n = 117, $P < 0.05$ versus distal colon for Gt and Pd).

STa increased Isc and Pd without significantly altering Gt (table 1). The latency of the Isc response was 2 min, and a plateau was reached after 10-15 min, which was quite stable for at least 60 min. The action of STa could be terminated by replacing the mucosal compartment with fresh buffer solution.

3.2. Action of TTX

Tetrodotoxin (1.25 μM added to the serosal side) had no significant effect on the response to STa in the colon. In the distal colon the response to STa (100 $U \cdot ml^{-1}$) was 36.3 ± 5.7 $\mu A \cdot cm^{-2}$ in the absence and 31.4 ± 9.6 $\mu A \cdot cm^{-2}$ in the presence of TTX (n = 5, difference not significant). In the proximal colon STa

TABLE 1

Electrical parameters, unidirectional and net ion fluxes of Na^+ and Cl^-. Fluxes were measured in the absence of any drugs (control), in a period, where only TTX (1.25 μM on the serosal side) was present, 20 min after addition STa (6 and 25 $U \cdot ml^{-1}$ on the mucosal side for proximal and distal segments, respectively), and 15 min after addition of indomethacin (1 μM on both sides). Distal (top) and proximal (bottom) colon. For better comparability, Isc is expressed in $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ (1 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ equals 26.9 $\mu A \cdot cm^{-2}$). Values are means ± S.E., n = 7-8.

Period	Isc ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)	Gt ($mS \cdot cm^{-2}$)	Pd (mV)	J_{ms}^{Na}	J_{sm}^{Na}	J_{net}^{Na} ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)	J_{ms}^{Cl}	J_{sm}^{Cl}	J_{net}^{Cl}	J_{net}^{R}
Distal colon										
Control	1.9 ± 0.3	8.8 ± 0.5	6.2 ± 0.8	15.9 ± 1.0	5.1 ± 0.3	10.8 ± 1.1	24.4 ± 1.1	17.2 ± 1.1	7.1 ± 1.6	−1.7 ± 1.9
TTX	1.2 ± 0.1 [a]	8.0 ± 0.4	3.9 ± 0.3	16.8 ± 0.5	4.8 ± 0.3	12.0 ± 0.6	22.8 ± 1.4 [a]	14.2 ± 1.7 [a]	8.6 ± 2.2	−2.3 ± 3.7
STa	2.4 ± 0.2 [a]	8.5 ± 0.6	7.5 ± 0.5 [a]	11.8 ± 0.9 [a]	4.2 ± 0.2	7.6 ± 1.0	15.6 ± 1.4 [a]	14.3 ± 1.1	1.2 ± 1.8	−4.0 ± 2.1
Indomethacin	0.8 ± 0.1 [a]	6.6 ± 0.5	3.6 ± 0.4 [a]	12.6 ± 1.4	3.5 ± 0.4	9.1 ± 1.4	19.2 ± 1.4	9.5 ± 1.5 [a]	9.6 ± 2.0	1.3 ± 2.5
Proximal colon										
Control	2.5 ± 0.2	32.0 ± 3.4	2.4 ± 0.3	17.4 ± 1.3	11.2 ± 2.2	6.2 ± 2.5	14.8 ± 2.3	25.5 ± 3.0	−10.7 ± 3.7	−14.3 ± 4.5
TTX	2.1 ± 0.2	28.8 ± 3.3	2.2 ± 0.2	15.7 ± 1.2	10.0 ± 1.4	5.7 ± 1.9	11.1 ± 1.1	22.4 ± 1.5	−11.4 ± 1.9	−14.9 ± 2.7
STa	5.0 ± 0.3 [a]	29.3 ± 2.6	5.1 ± 0.4 [a]	10.8 ± 1.3 [a]	8.6 ± 1.1	2.2 ± 1.7	12.2 ± 2.2	30.6 ± 2.2 [a]	−18.4 ± 3.1	−15.7 ± 3.5
Indomethacin	4.2 ± 0.2	25.8 ± 2.3	4.8 ± 0.4 [a]	10.1 ± 0.9	6.7 ± 0.9	3.4 ± 1.3	14.6 ± 2.3	26.8 ± 1.2	−12.2 ± 2.6	−11.4 ± 3.2

[a] $P < 0.05$ versus preceding period.

(25 $U \cdot ml^{-1}$) increased Isc by 35.3 ± 90 $\mu A \cdot cm^{-2}$ in the absence of TTX and by 33.0 ± 8.9 $\mu A \cdot cm^{-2}$ in the presence of the neuronal blocker (n = 5, difference not significant).

3.3. Unidirectional flux measurements

Tetrodotoxin caused a significant decrease of Isc, J_{ms}^{Cl} and J_{sm}^{Cl} in the distal colon, but had no significant effects in the proximal colon (table 1). In the distal colon in the presence of TTX the mucosa to serosa fluxes of Na^+ and Cl^- exceeded the corresponding serosa to mucosa fluxes. The consequence was absorption of Na^+ and Cl^- (table 1) and the positive Isc of 1.2 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ (equals 32.3 $\mu A \cdot cm^{-2}$) represents mainly the difference between J_{net}^{Na} and J_{net}^{Cl}.

In the proximal colon, the mucosa to serosa flux of Na^+ was higher than the serosa to mucosa flux. The situation was the reverse for Cl^-: J_{sm}^{Cl} exceeded J_{ms}^{Cl}. Consequently, Na^+ absorption went on simultaneously with Cl^- secretion (table 1). In this segment J_{net}^{R}, the flux of all ions other than Na^+ and Cl^- was much greater than in the distal segment.

STa caused a significant decrease of the mucosa to serosa fluxes of Na^+ and of Cl^- in the distal colon, but had no effect on J_{sm}^{Na} or J_{sm}^{Cl} (table 1). J_{net}^{Cl} was inhibited to a greater extent than J_{net}^{Na}. The increasing difference between the net fluxes of Na^+ and Cl^- was probably responsible for the increase in Isc after addition of STa. In contrast, in the proximal colon, STa decreased the mucosa to serosa flux of Na^+ and increased the serosa to mucosa flux of Cl^- significantly. The consequence was an increase in Cl^- secretion and an increase in Isc.

Indomethacin (1 μM at the mucosal and the serosal side), when added at the plateau in Isc induced by STa, decreased significantly the Isc response to STa to a value below the former baseline in the distal colon. Indomethacin caused an (insignificant) increase in J_{ms}^{Na} and J_{ms}^{Cl} and decreased significantly the serosa to mucosa flux of Cl^-. The consequence was recovery of Na^+ and Cl^- absorption; the values approached that for the period before addition of STa. In contrast, in the proximal colon, indomethacin had no significant effect on electrical parameters or on unidirectional ion fluxes (table 1).

3.4. Ionic dependence of the STa response

Replacement of Cl^- ions by gluconate decreased the baseline Isc from 63.8 ± 10.6 to 21.3 ± 4.3 $\mu A \cdot cm^{-2}$ in the distal colon (n = 6, $P < 0.05$). The response to STa was decreased by 85% in the Cl^--free medium (n = 6, table 2). In the proximal colon, the baseline was 34.0 ± 11.5 $\mu A \cdot cm^{-2}$ in Cl^--containing solution and -4.2 ± 2.9 $\mu A \cdot cm^{-2}$ in Cl^--free solution (n = 5, $P < 0.05$). The STa response was decreased by about 80% under Cl^--free conditions in this segment (n = 5, table 2).

TABLE 2

Effect of transport inhibitors and anion replacement on the response to STa. The increase in Isc induced by STa (100 and 30 $U \cdot ml^{-1}$ in the distal and the proximal colon, respectively) was tested first under control conditions then in Cl^-- or HCO_3^--free solution, in the presence of NPPB (100 μM on the mucosal side), furosemide (100 μM on the serosal side), SITS (1 mM on the mucosal side), or amiloride (1 mM on the mucosal side). The values are given as differences from the former baseline and are means ± S.E., n = 5-7.

	Distal colon Δ Isc to STa ($\mu A \cdot cm^{-2}$)	Proximal colon Δ Isc to STa ($\mu A \cdot cm^{-2}$)
Control	56.3 ± 10.0	53.2 ± 11.4
Cl^--free	7.3 ± 1.2 [a]	11.0 ± 4.3 [a]
Control	43.6 ± 6.6	29.3 ± 3.2
HCO_3^--free	21.2 ± 3.9 [a]	13.0 ± 3.8 [a]
Control	31.6 ± 7.7	59.3 ± 14.3
NPPB	1.2 ± 1.2 [a]	12.6 ± 3.3 [a]
Control	53.7 ± 11.9	102.2 ± 21.0
Furosemide	17.0 ± 2.2 [a]	64.8 ± 10.2 [a]
Control	48.7 ± 7.0	77.2 ± 7.8
SITS	48.1 ± 1.5	70.9 ± 2.8
Control	55.6 ± 5.7	42.3 ± 7.9
Amiloride	46.7 ± 10.2	38.5 ± 5.9

[a] $P < 0.05$ versus response to STa under control conditions.

The STa response was also partially dependent on the presence of bicarbonate ions. In HCO_3^--free buffer, the baseline Isc in the distal colon was decreased from 95.7 ± 18.1 to 56.9 ± 9.0 $\mu A \cdot cm^{-2}$ (n = 7, $P < 0.05$). In the proximal colon, omission of HCO_3^- had no significant effect on baseline Isc (48.8 ± 15.3 $\mu A \cdot cm^{-2}$ in the presence and 33.8 ± 15.3 $\mu A \cdot cm^{-2}$ in the absence of HCO_3^-, n = 6). Omission of HCO_3^- caused a decrease of 50% of the effect of STa in both colonic segments (n = 6-7, table 2).

The Cl^- channel blocker, NPPB (100 μM; Wangemann et al., 1986), decreased the STa response in both distal and proximal colon by around 80% (n = 5-7, table 2). Furosemide (100 μM), the inhibitor of the Na^+-K^+-$2Cl^-$ co-transporter, led to a decrease of the STa response by 60 and 37% in the distal and the proximal colon, respectively (n = 6, table 2).

Amiloride in a concentration of 1 mM, which is known to inhibit Na^+/H^+ exchange (for references see Benos, 1982), was without effect on the increase in Isc due to STa in both distal and proximal colon (n = 6-7, table 2). In both colonic segments the blockade of Cl^-/HCO_3^- exchange by SITS (1 mM) did not change the increase in Isc due to STa (n = 5-6, table 2).

3.5. Involvement of Ca^{2+}

Verapamil, a Ca^{2+} channel blocker, TMB-8, an inhibitor of the Ca^{2+} release from intracellular stores,

TABLE 3

Effect of inhibitors of the Ca^{2+} pathway on the response to STa. The increase in Isc induced by STa (50 and 6 $U \cdot ml^{-1}$, in distal and proximal colon, respectively) was tested first under control conditions, then in the absence of serosal or mucosal Ca^{2+}, or in the presence of verapamil (50 μM on the serosal side), TMB-8 (10 μM on the serosal side), or trifluoperazine (100 μM on the serosal side). The values are given as differences from the former baseline and are means ± S.E., n = 4-11.

	Distal colon Δ Isc to STa ($\mu A \cdot cm^{-2}$)	Proximal colon Δ Isc to STa ($\mu A \cdot cm^{-2}$)
Control	61.9 ± 18.0	180.7 ± 23.9
Serosal Ca^{2+}-free	72.2 ± 16.8	189.7 ± 26.3
Control	33.8 ± 7.1	100.5 ± 21.2
Mucosal Ca^{2+}-free	28.1 ± 8.0	90.8 ± 19.8
Control	31.4 ± 5.1	158.4 ± 19.2
Verapamil	38.1 ± 9.1	147.9 ± 24.0
Control	24.4 ± 5.3	98.3 ± 15.2
TMB-8	22.7 ± 4.4	98.2 ± 13.2
Control	33.5 ± 7.2	99.9 ± 21.6
Trifluoperazine	27.5 ± 8.0	75.2 ± 18.8

and trifluoperazine, an inhibitor of the Ca^{2+}-dependent regulator protein, calmodulin, were unable to decrease the response to STa in both segments. The removal of Ca^{2+} from the mucosal or the serosal compartment was also without effect on the response to STa (n = 5-7, table 3). Consequently, the action of STa is independent of external and internal Ca^{2+} ions.

3.6. Involvement of prostaglandins

The cyclooxygenase inhibitor, indomethacin (1 μM), abolished the response to STa when added during the plateau phase of the Isc response to STa in the distal colon (table 1). Also when used for pretreatment, indomethacin inhibited the STa response in the distal colon. STa (100 $U \cdot ml^{-1}$ on the mucosal side) increased Isc by 32.0 ± 5.0 $\mu A \cdot cm^{-2}$ in the absence and by 13.5 ± 2.0 $\mu A \cdot cm^{-2}$ in the presence of indomethacin (n = 8, $P < 0.05$, fig. 2). In this segment, indomethacin decreased the baseline Isc by 37.0 ± 0.6 $\mu A \cdot cm^{-2}$ (n = 31, $P < 0.05$).

In the proximal colon, indomethacin pretreatment was without any effect on the response to STa. STa (12 $U \cdot ml^{-1}$ added on the mucosal side) caused an increase of Isc of 29.4 ± 4.5 $\mu A \cdot cm^{-2}$ in the absence and of 31.2 ± 4.7 $\mu A \cdot cm^{-2}$ in the presence of indomethacin (n = 8, difference not significant versus response in the absence of indomethacin). In the proximal colon, indomethacin decreased the baseline Isc by 86.1 ± 1.2 $\mu A \cdot cm^{-2}$ (n = 38, $P < 0.05$).

The response to 8-Br-cyclic GMP, like that to STa, varies along the longitudinal axis of the colon; the distal colon is less sensitive than the proximal colon (Nobles et al., 1991). In the distal colon the action of

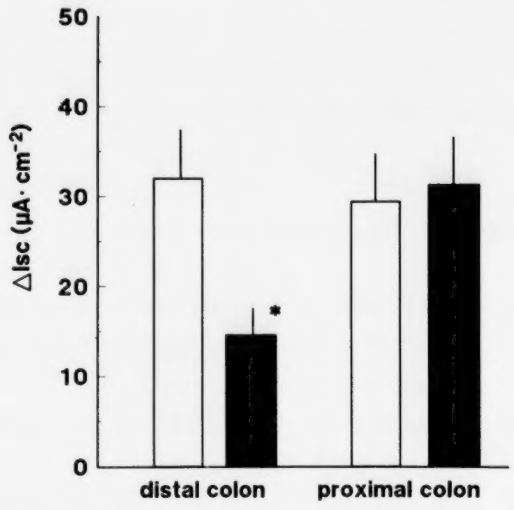

Fig. 2. Action of STa (100 and 12 $U \cdot ml^{-1}$ in the distal and the proximal colon, respectively) in the absence (white bars) and in the presence of indomethacin (1 μM on the mucosal and the serosal side, black bars). Distal (left) and proximal (right) rat colon. The values are means ± S.E., n = 8, * $P < 0.05$ versus STa alone.

8-Br-cyclic GMP on Isc was inhibited by 75% by indomethacin (n = 7, table 4). In contrast, indomethacin had no effect on the electrical response to 8-Br-cyclic GMP in the proximal colon (table 4).

The inhibition of the STa response by indomethacin could be overcome by PGE_2. Under control conditions STa (100 $U \cdot ml^{-1}$) caused an increase of 28.7 ± 2.6 $\mu A \cdot cm^{-2}$. In the presence of indomethacin the response to the toxin was decreased to 6.7 ± 1.2 $\mu A \cdot cm^{-2}$ (n = 6, $P < 0.05$ versus response under control conditions). When PGE_2 was added in a concentration of 50 nM, it elevated Isc by 59.3 ± 4.4 $\mu A \cdot cm^{-2}$ (n = 6, $P < 0.05$). In the combined presence of both indomethacin and PGE_2, the response to STa was 26.3 ± 3.9 $\mu A \cdot cm^{-2}$. This response was not significantly different from the initial Isc response in the absence of indomethacin (figs. 3, 4).

Forskolin, an activator of adenylate cyclase (Seamon et al., 1981), also completely restored the action of STa (fig. 4), suggesting that the dependence of the STa response on prostaglandins involves cyclic AMP. Forskolin (500 nM) itself led to an increase of Isc of 48.7 ± 5.1 $\mu A \cdot cm^{-2}$ (n = 8, $P < 0.05$) in these experi-

TABLE 4

Action of 8-Br-cyclic GMP on Isc in the absence and in the presence of indomethacin (1 μM on the mucosal and the serosal side). 8-Br-cyclic GMP was added on the serosal side in a concentration of 500 μM in the distal colon and at a concentration of 100 μM in the proximal colon. The values are given as differences from the baseline and are means ± S.E., n = 5-6.

	Δ Isc ($\mu A \cdot cm^{-1}$) 8-Br-cyclic GMP alone	8-Br-cyclic GMP + indomethacin
Distal colon	35.3 ± 8.5	17.0 ± 4.3 [a]
Proximal colon	61.4 ± 5.8	93.6 ± 12.9

[a] $P < 0.05$ versus response to 8-Br-cyclic GMP alone.

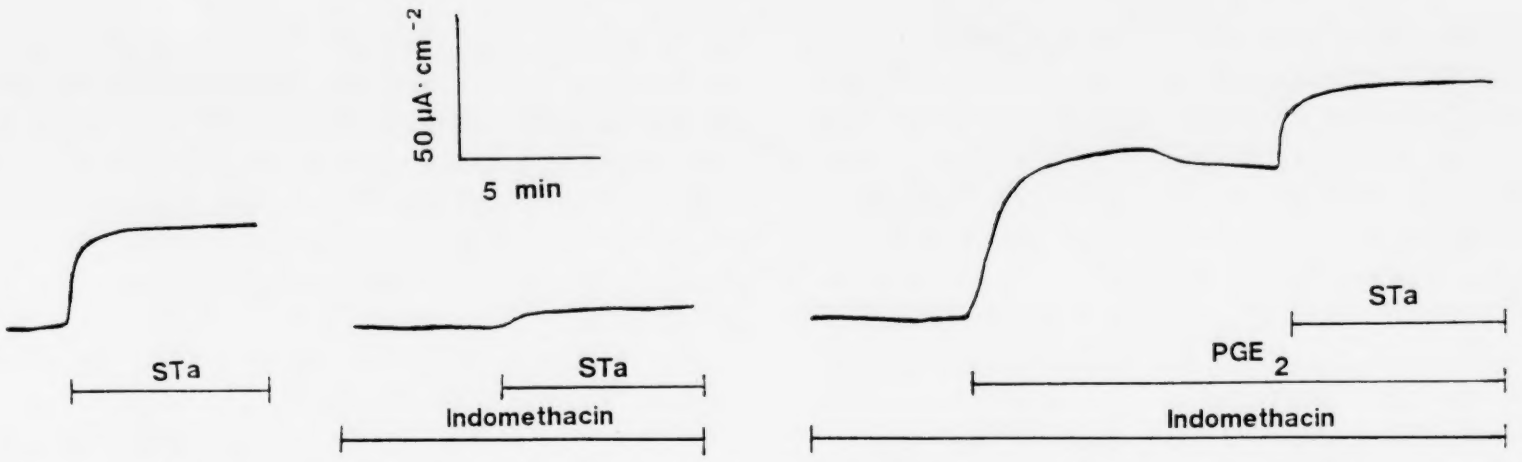

Fig. 3. Recovery of the STa response by PGE_2 after inhibition by indomethacin. Distal colon. The response to STa (100 $U \cdot ml^{-1}$ on the mucosal side) was first tested in the absence of indomethacin, then in the presence of indomethacin (1 μM on the mucosal and the serosal side), and finally in the presence of both indomethacin and PGE_2 (50 nM on the serosal side). The original record is representative of six experiments with similar results, for statistics see fig. 4.

ments. In contrast to PGE_2 or forskolin, CPT-cyclic AMP, a membrane-permeable analogue of cyclic AMP, did not restore the response to STa after addition of indomethacin, although CPT-cyclic AMP (25 μM) increased Isc by 48.2 ± 4.9 $\mu A \cdot cm^{-2}$ ($n = 6$, $P < 0.05$; not statistically different from the action of PGE_2 or forskolin). The response to STa was 30.8 ± 3.4 $\mu A \cdot cm^{-2}$ under control conditions, 17.0 ± 2.3 $\mu A \cdot cm^{-2}$ in indomethacin-treated tissues ($n = 6$, $P < 0.05$ versus control) and 4.0 ± 2.6 $\mu A \cdot cm^{-2}$ in the presence of both indomethacin and CPT-cyclic AMP ($n = 6$, $P < 0.05$ versus control, fig. 4). The Isc response evoked by CPT-cyclic AMP was not enhanced by IBMX (100 μM), a non-specific inhibitor of phosphodiesterases, suggesting that this nucleotide is probably not hydrolysed in the colon (data not shown).

3.7. Involvement of a phosphodiesterase

It is known from tissues such as the heart or platelets, that there is an interaction between cyclic AMP and

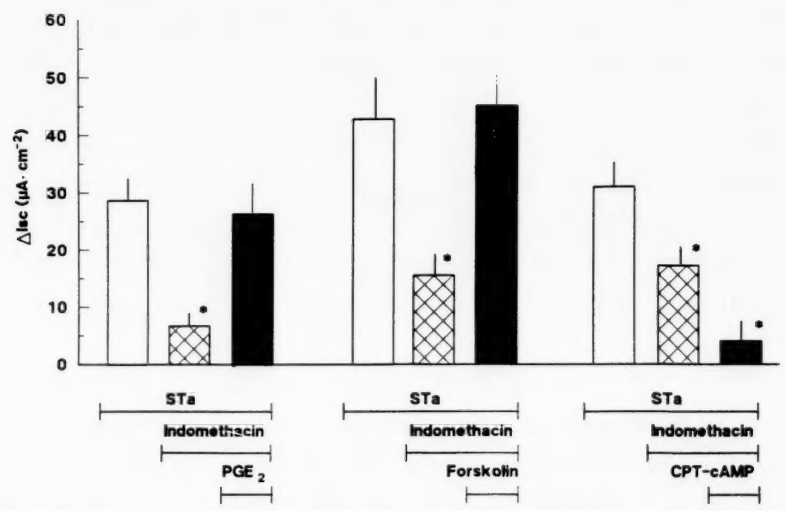

Fig. 4. Action of forskolin (500 nM, on the serosal and the mucosal side), PGE_2 (50 nM on the serosal side), and CPT-cyclic AMP (25 μM on the serosal side) on the response to STa (75 $U \cdot ml^{-1}$ on the mucosal side) inhibited by 1 μM indomethacin. Distal colon. The experimental protocol was the same as in fig. 3. An analysis of variance revealed that the increase in Isc induced by the agonists (see text) was not statistically different. The values are means $\pm$S.E., $n = 6$-8, * $P < 0.05$ versus STa alone.

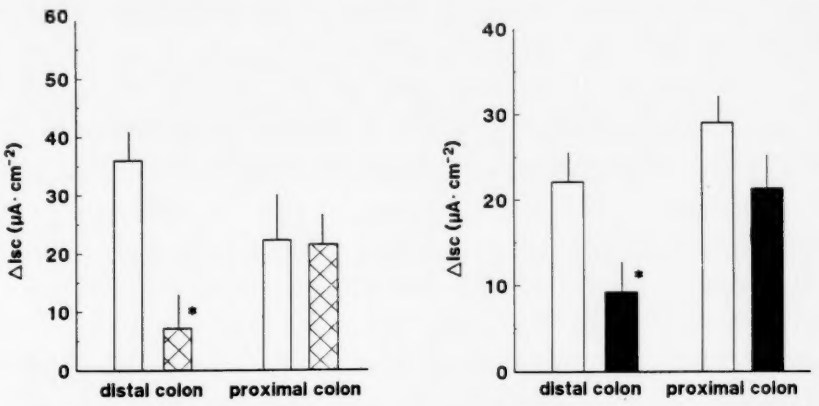

Fig. 5. Effect of STa (50 and 25 $U \cdot ml^{-1}$ in distal and proximal colon, respectively) in the absence (white bars) and in the presence of amrinone (100 μM on the serosal side, left) or trequinsin (1 μM on the serosal side, right). The values are means $\pm$S.E., $n = 6$-8, * $P < 0.05$ versus STa alone.

cyclic GMP involving a phosphodiesterase, which hydrolyses cyclic AMP and which can be inhibited by cyclic GMP (Beavo, 1988). In order to test the involvement of a cyclic GMP-inhibited phosphodiesterase in the response to STa, the effects of amrinone and trequinsin, two inhibitors of this type of phosphodiesterases, were studied.

Amrinone (100 μM) increased the Isc by 27.5 ± 2.2 and 22.2 ± 4.1 $\mu A \cdot cm^{-2}$ in distal and proximal colon, respectively ($n = 6$, $P < 0.05$). Amrinone led to an inhibition of 80% of the STa response in the distal colon ($n = 6$, $P < 0.05$, fig. 5), but had no significant effect in the proximal colon. Trequinsin (1 μM) increased the Isc by 27.0 ± 7.6 and 16.4 ± 2.4 $\mu A \cdot cm^{-2}$ in distal and proximal colon, respectively. Trequinsin led to a decrease of 58% of the response to STa in distal colon ($n = 7$, $P < 0.05$, fig. 5), but had no effect on the response to STa in the proximal colon.

4. Discussion

The STa induced an increase of Isc in the rat colon (fig. 1). The maximal response to STa was higher in the

proximal colon than in the distal colon. Unidirectional flux measurements revealed that there were not only quantitative, but also qualitative differences in the response to STa along the longitudinal axis of the colon (table 1). STa activated the secretory, i.e. serosa to mucosa flux of Cl^- in the proximal colon and decreased the absorptive, i.e. mucosa to serosa flux of Na^+. In contrast, in the distal colon it had no effect on J_{sm}^{Cl} but inhibited J_{ms}^{Na} and J_{ms}^{Cl}. Consequently, STa inhibited only the net absorption of Na^+ and Cl^- in the distal colon, but enhanced the spontaneously active Cl^- secretion in the proximal colon. In both segments, J_{net}^{Cl} was affected to a greater extent than J_{net}^{Na}. In the distal colon, this difference between J_{net}^{Na} and J_{net}^{Cl} is responsible for the increase in Isc. Similar discrepancies, concomitant with an increase in Isc, have been observed with the changes in ion transport induced by a sea-anemone toxin (Bridges et al., 1986), with the Cl^- channel blocker, NPPB (Diener and Rummel, 1989) and also with furosemide (see below). Although transepithelial NaCl absorption in the rat colon is an electroneutral process, involving electrically silent ion exchangers in the apical membrane, the basolateral exit of Na^+ (via the $3Na^+$-$2K^+$-ATPase) is electrogenic. Thus the basolateral exit of Cl^- must also be mediated by an electrogenic process as discussed in detail elsewhere (Diener et al., 1989). Consequently, all manoeuvres that will affect Na^+ and Cl^- transport to different extents will induce a change in Isc.

The results of the anion replacement experiments supported the assumption that Cl^- is the main ion responsible for the electrical response to STa. The effect of STa was diminished by 90% in the proximal and by 75% in the distal colon when Cl^- was omitted from the extracellular medium (table 2). Bicarbonate anions also seem to participate in the electrical response to STa because the removal of HCO_3^- decreased the STa response by about 50% in both segments (table 2). A possible mechanism of action might be the inhibition of Cl^- absorption after removal of HCO_3^- (Binder et al., 1987), which will impair the ability of STa to inhibit Cl^- absorption.

Chloride secretion if thought to be mediated via a basolateral Na^+-K^+-$2Cl^-$ cotransporter, which can be blocked by loop diuretics, and via apical Cl^- channels (Frizzell and Heintze, 1979). Surprisingly, the electrical response to STa can be inhibited by the Cl^- channel blocker, NPPB (Wangemann et al., 1986), and by the loop diuretic, furosemide, not only in the proximal colon as one could have expected, but also in the distal colon, where STa did not induce Cl^- secretion (table 2). This probably reflects unspecific actions of these inhibitors. NPPB is known to exert, besides its antisecretory action via blockade of apical Cl^- channels, an inhibitory action on NaCl absorption (Diener and Rummel, 1989). Furosemide, when added to control tissue with a low baseline, also induces a paradox increase in Isc (Diener and Rummel, 1990) which is associated with an inhibition of J_{ms}^{Na} and J_{ms}^{Cl} (Diener and Rummel, unpublished observations). Consequently, both drugs might interfere with the inhibition of absorption by STa and might thus also be able to decrease the Isc response evoked by the toxin in the distal colon.

The action of STa was not dependent on intra- or extracellular Ca^{2+}. Omission of Ca^{2+} from the medium, addition of a Ca^{2+} channel blocker, addition of TMB-8, an inhibitor of Ca^{2+} release from intracellular stores (Malagodi and Chiou, 1974), or addition of the calmodulin antagonist, trifluoperazine, did not affect the Isc response to STa in the distal or the proximal colon. This is in contrast to mouse small intestine, where inhibitors of the Ca^{2+} pathway have been shown to decrease the secretory response to STa (Abbey and Knoop, 1979; Greenberg et al., 1980, 1982). Segment and/or species differences may be responsible for this discrepancy.

The secretory action of STa is thought to be mediated by cyclic GMP (Field et al., 1978; Hughes et al., 1978). Consistent with this assumption, the membrane-permeable cyclic GMP-analogue, 8-Br-cyclic GMP, presented the same heterogeneity in its action on the rat colon as did STa. The proximal colon was more sensitive and showed a higher maximal increase in Isc than the distal colon (Nobles et al., 1991; see also table 4). Obviously, due to the intracellular action of 8-Br-cyclic GMP, these results suggest that a difference in the density of STa receptors between the proximal and the distal colon cannot be the reason for the different sensitivity of STa.

A role has been proposed for prostaglandins in the mediation of the secretory response to STa in the small intestine, because the cyclooxygenase blocker, indomethacin, used at a very high concentration (1 mM), inhibits the action of STa (Madsen and Knoop, 1978; Greenberg et al., 1980; Guerrant et al., 1980). Prostaglandins are known to be involved in the modulation of electrolyte transport. They inhibit intestinal absorption and activate secretion (Al-Awqati and Greenough, 1972). Their action on electrolyte transport is mediated by a direct activation of the epithelium via receptor-mediated stimulation of adenylate cyclase (Smith et al., 1987) and indirectly by stimulation of submucosal neurons (Diener et al., 1988). In the experiments presented here, which were carried out in the presence of the neurotoxin, TTX, only the epithelial actions of prostaglandins need be considered.

Both the action of STa and the effect of 8-Br-cyclic GMP were inhibited by indomethacin in the distal colon, but not in the proximal colon. Inhibition was obtained with a low concentration of indomethacin (1 μM) and could be overcome by PGE_2 (figs. 3, 4). Both

results strongly suggest a role for prostaglandins in the mediation of the Isc response to STa. Inhibition of the action of the cyclic GMP analogue indicates that the action of prostaglandins is localized at a step that follows the activation of particulate guanylate cyclase, i.e. distal from the coupling between the STa receptor and guanylate cyclase. The problem of coupling may be resolved by a recent molecular biological study, which has revealed that the intestinal membrane-bound guanylate cyclase itself can serve as the STa receptor (Schulz et al., 1990).

The inhibition of the STa response could also be overcome by forskolin (fig. 4), an activator of adenylate cyclase (Seamon et al., 1981; Bridges et al., 1983), indicating that this prostaglandin action is probably mediated by cyclic AMP. Surprisingly, CPT-cyclic AMP, a non-hydrolysable analogue of cyclic AMP, did not restore the action of STa (fig. 4). What remains is the hypothesis that the hydrolysis of cyclic AMP may be the crucial point.

Different phosphodiesterases can be involved in the interactions of cyclic GMP with the hydrolysis of cyclic AMP. Both cyclic GMP-inhibited and cyclic GMP-activated phosphodiesterases have been described (for references see Beavo 1988; Beavo and Reifsnyder, 1990). Inhibition by cyclic GMP of a phosphodiesterase responsible for the degradation of cyclic AMP would lead to a secondary increase in the intracellular cyclic AMP concentration. Consequently, the action of STa (and cyclic GMP) would be dependent on the intracellular level of cyclic AMP, which is controlled by prostaglandins (Smith et al., 1987). In the rat colon in vitro, there is a spontaneous production of eicosanoids, which activates Isc even under control conditions (Cuthbert and Margolius, 1982; Diener et al., 1988). This secretory tone is abolished by indomethacin and consequently, an – indirect – inhibition of the action of STa could take place.

This hypothesis is supported by the results obtained with amrinone and trequinsin, two blockers of cyclic GMP-inhibited phosphodiesterases in the heart (Ruppert and Weithmann, 1982; Harrison et al., 1986). Both drugs mimicked the action of STa in the colon, i.e. they caused an increase in Isc in the proximal and the distal colon, suggesting that there exists in both parts of the large intestine a cyclic AMP phosphodiesterase which is controlled by cyclic GMP. Amrinone and trequinsin prevented the action of a subsequent administration of STa only in the distal colon. Thus, these drugs may share a common intracellular target with STa, i.e. a cyclic GMP-inhibited phosphodiesterase in the distal colon (fig. 6). Consequently, in the distal colon, cyclic AMP is the intracellular messenger acting on membrane transporters involved in the STa response. To find whether the action of cyclic AMP is mediated by a cyclic AMP-dependent protein kinase should be the aim of further investigations. Modulation of a cyclic GMP-inhibited phosphodiesterase in some intestinal segments may be a new explanation for the long-documented inhibition of the STa response in several intestinal segments by indomethacin (Madsen and Knoop, 1978; Greenberg et al., 1980; Guerrant et al., 1980).

In contrast, there is no inhibition by indomethacin and consequently no indication of an involvement of prostaglandins in the generation of the secretory response to STa in the proximal colon. A cyclic GMP-dependent protein kinase has been found in the small intestine. The incubation of tissue with cyclic GMP is

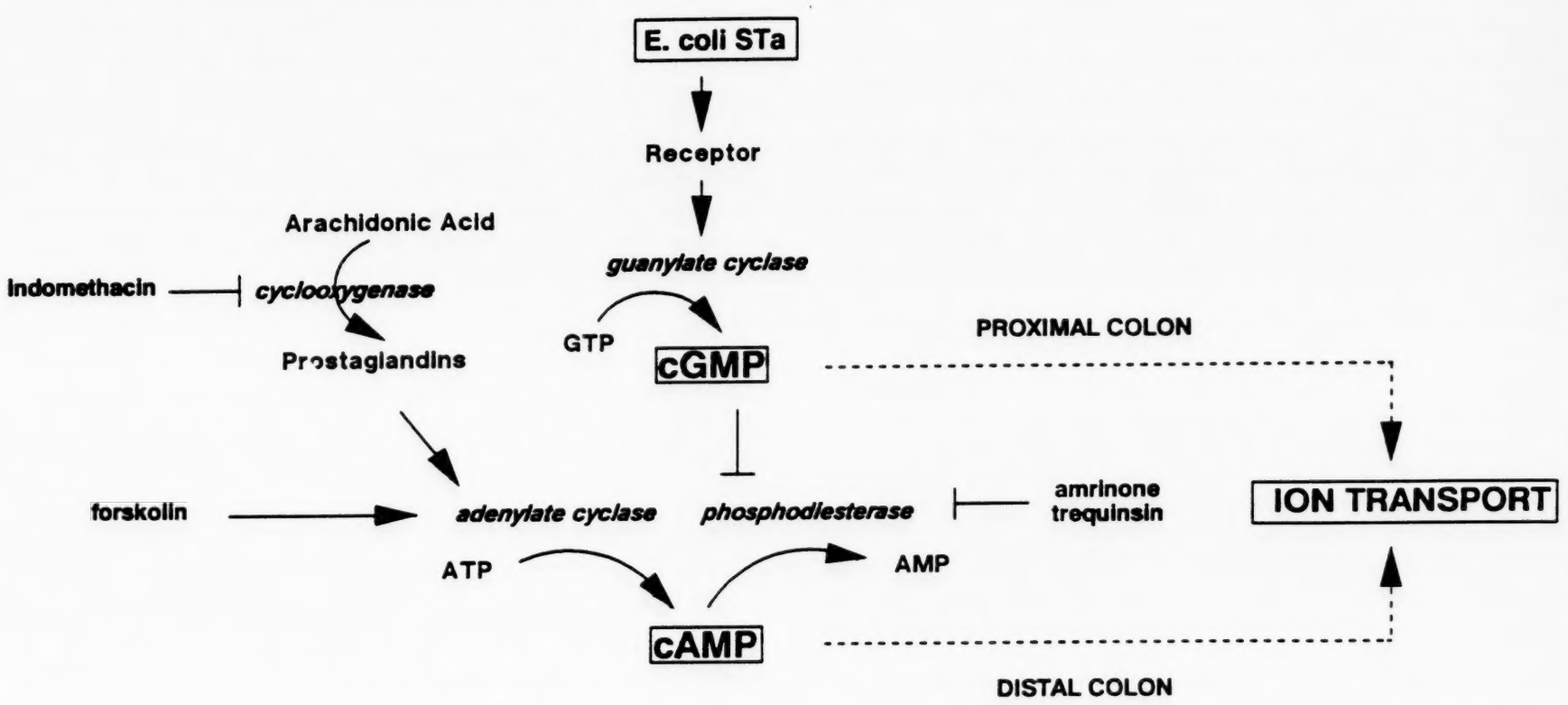

Fig. 6. Model proposed for the intracellular action of STa in the distal and the proximal colon.

followed by phosphorylation of membrane proteins (De Jonge, 1981; for review see Waldman and Murad, 1987). Phosphorylation of ion transporters (and/or regulator proteins) or direct actions of the nucleotide on ion channels are possible candidates for the mediation of the STa response in this segment of the colon (fig. 6).

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References

Abbey, F.M. and F.C. Knoop, 1979, Effect of chlorpromazine on the secretory activity of *Escherichia coli* heat-stable enterotoxin, Infect. Immun. 26, 1000.

Al-Awqati, Q. and W.B. Greenough, 1972, Prostaglandins inhibit intestinal sodium transport, Nature/New Biol. 238, 26.

Andres, H., R. Bock, R.J. Bridges, W. Rummel and J. Schreiner, 1985, Submucosal plexus and electrolyte transport across rat colonic mucosa, J. Physiol. 364, 301.

Beavo, J.A., 1988, Multiple isozymes of cyclic nucleotide phosphodiesterase, in: Advances in Second Messenger and Phosphoprotein Research, Vol. 22, eds. P. Greengard and G.A. Robinson (Raven Press, New York) p. 1.

Beavo, J.A. and D.H. Reifsnyder, 1990, Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors, Trends Pharmacol. Sci. 11, 150.

Benos, D.J., 1982, Amiloride: a molecular probe of sodium transport in tissues and cells, Am. J. Physiol. 242, C131.

Binder, H.J., E.S. Foster, M.E. Budinger and J.P. Hayslett, 1987, Mechanism of electroneutral sodium chloride absorption in distal colon of the rat, Gastroenterology 93, 449.

Bridges, R.J., W. Rummel and B. Simon, 1983, Forskolin induces chloride secretion across the isolated mucosa of rat colon descendens, Naunyn-Schmiedeb. Arch. Pharmacol. 323, 355.

Bridges, R.J., M. Rack, W. Rummel and J. Schreiner, 1986, Mucosal plexus and electrolyte transport across the rat colonic mucosa, J. Physiol. 376, 531.

Cuthbert, A.W. and H.S. Margolius, 1982, Kinins stimulate net chloride secretion by the rat colon, Br. J. Pharmacol. 75, 587.

De Jonge, H.R., 1981, Cyclic GMP-dependent protein kinase in intestinal brushborders, in: Adv. Cycl. Nucl. Res. 14, eds: E. Dumont, P. Greengard and G.A. Robinson (Raven Press, New York) p. 315.

De Jonge, H.R., 1984, The mechanism of action of *Escherichia coli* heat-stable toxin, Biochem. Soc. Trans. 12, 180.

Diener, M. and W. Rummel, 1989, Actions of the chloride channel blocker NPPB on absorptive and secretory transport processes in rat colon, Acta Physiol. Scand. 137, 215.

Diener, M. and W. Rummel, 1990, Distension-induced secretion in the rat colon: mediation by prostaglandins and submucosal neurons, Eur. J. Pharmacol. 178, 47.

Diener, M., R.J. Bridges, S.F. Knobloch and W. Rummel, 1988, Neuronally mediated and direct effects of prostaglandins on ion transport in rat colon descendens, Naunyn-Schmiedeb. Arch. Pharmacol. 337, 74.

Diener, M., W. Rummel, P. Mestres and B. Lindemann, 1989, Single chloride channels in colon mucosa and isolated colonic enterocytes of the rat, J. Membr. Biol. 108, 21.

Dreyfus, L.A., L. Jaso Friedman and D.C. Robertson, 1984, Characterization of the mechanism of action of *Escherichia coli* heat-stable enterotoxin, Infect. Immun. 44, 493.

Eklund, S., M. Jodal and O. Lundgren, 1985, The enteric nervous system participates in the secretory response to the heat-stable enterotoxins of *Escherichia coli* in rats and cats, Neuroscience 14, 673.

Field, M., L.H. Graf, W.J. Laird and P.L. Smith, 1978, Heat-stable enterotoxin of *Escherichia coli*: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine, Proc. Natl. Sci. U.S.A. 75, 2800.

Foster, E.S., M.E. Budinger, J.P. Hayslett and H.J. Binder, 1986, Ion transport in proximal colon of the rat. Sodium depletion stimulates neutral sodium chloride absorption, J. Clin. Invest. 77, 228.

Frizzell, R.A. and K. Heintze, 1979, Electrogenic chloride secretion by mammalian colon, in: Mechanisms of Intestinal Secretion, ed. H.J. Binder (Alan R. Liss, New York) p. 101.

Gebhardt, U., 1979, Exakte Kompensation des Lösungswiderstandes bei Anwendung der Voltage-Clamp Methode an Darmepithelien, (Thesis, Universität des Saarlandes, Homburg/Saar).

Giannella, R.A., 1981, Pathogenesis of acute bacterial diarrheal disorders, Ann. Rev. Med. 32, 341.

Giannella, R.A., M. Luttrell and M. Thompson, 1983, Binding of *Escherichia coli* heat-stable enterotoxin to receptors on rat intestinal cells, Am. J. Physiol. 245, G492.

Greenberg, R.N. and R.L. Guerrant, 1981, *E. coli* heat-stable enterotoxin, Pharmacol. Ther. 13, 507.

Greenberg, R.N., F. Murad, B. Chang, D.C. Robertson and R.L. Guerrant, 1980, Inhibition of *Escherichia coli* heat-stable enterotoxin by indomethacin and chlorpromazine, Infect. Immun. 29, 908.

Greenberg, R.N., F. Murad and R.L. Guerrant, 1982, Lanthanum chloride inhibition of the secretory response to *Escherichia coli* heat-stable enterotoxin, Infect. Immun. 35, 483.

Guerrant, R.L., J.M. Hughes, B. Chang, D.C. Robertson and F. Murad, 1980, Activation of intestinal guanylate cyclase by heat-stable enterotoxin of *Escherichia coli*: Studies of tissue specificity, potential receptors, and intermediates, J. Infect. Dis. 142, 220.

Harrison, S.A., D.H. Reifsnyder, B. Gallis, G.G. Cadd and J.A. Beavo, 1986, Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: A receptor for new cardiotonic drugs, Mol. Pharmacol. 29, 506.

Hughes, J.M., F. Murad, B. Chang and R.L. Guerrant, 1978, Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*, Nature 271, 755.

Madsen, G.L. and F.C. Knoop, 1978, Inhibition of the secretory activity of *Escherichia coli* heat-stable enterotoxin by indomethacin, Infect. Immun. 22, 143.

Malagodi, M.H. and C.Y. Chiou, 1974, Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studies in smooth muscles, Eur. J. Pharmacol. 27, 25.

Nobles, M., M. Diener, P. Mestres and W. Rummel, 1991, Segmental heterogeneity of the rat colon in the response to activators of secretion on the cAMP-, the cGMP- and the Ca^{2+}-pathway, Acta Physiol. Scand. 142 (in press).

Rao, M.C. and M. Field, 1984, Enterotoxins and ion transport, Biochem. Soc. Trans. 12, 177.

Rao, M.C., P.S. Guandalini and M. Field, 1980, Mode of action of heat-stable *Escherichia coli* enterotoxin. Tissue and subcellular specificities and role of cyclic GMP, Biochem. Biophys. Acta 632, 35.

Ruppert, D. and K.U. Weithmann, 1982, HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation in vitro, Life Sci. 31, 2037.

Schulz, S., C.K. Green, P.S.T. Yuen and D.L. Garbers, 1990, Guanylyl cyclase is a heat-stable enterotoxin receptor, Cell 63, 941.

Seamon, K.B., W. Padgett and J.W. Daly, 1981, Forskolin: unique diterpene activator of adenylate cyclase in membranes and intact cells, Proc. Natl. Acad. Sci. U.S.A. 78, 3363.

Smith, G., G. Warhurst, M. Lees and L. Turnberg, 1987, Evidence that PGE_2 stimulates intestinal epithelial adenylate cyclase by a receptor-mediated mechanism, Dig. Dis. Sci. 32, 71.

Van Driessche, W. and B. Lindemann, 1978, Low-noise amplification of voltage and current fluctuations arising in epithelia, Rev. Scient. Instr. 49, 52.

Waldman, S.A. and F. Murad, 1987, Cyclic GMP synthesis and function, Pharmacol. Rev. 39, 163.

Waldman, S.A., T. Kuno, Y. Kamisaki, L.Y. Chang, J. Gariepy, P. O'Hanley, G. Schoolnik and F. Murad, 1986, Intestinal receptors for heat-stable enterotoxin of *Escherichia coli* is tightly coupled to a novel form of particulate guanylate cyclase, Infect. Immun. 51, 320.

Waldman, S.A., T. Kuno, Y. Kamisaki, B. Chang, J. Gariepy and F. Murad, 1987, Cyclic GMP synthesis and function, Pharmacol. Rev. 39, 163.

Wangemann, P., M. Wittner, A. Di Stefano, H.C. Englert, H.J. Lang, E. Schlatter and R. Greger, 1986, Cl^--channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship, Pflügers Arch. 407, S128.

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Putative role of endogenous insulin in cysteamine-induced hypersecretion of gastric acid in rats

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The effects of cysteamine on gastric acid secretion and blood glucose levels were examined in rats. Cysteamine given subcutaneously in doses of 100–300 mg/kg, decreased the level of arterial blood glucose dose dependently, but had no effects on the level of the jugular venous blood glucose. Glucose uptake into the brain, as obtained by subtracting level of the jugular venous blood glucose from level of the arterial blood glucose, was significantly decreased by the administration of cysteamine. The uptake of [^{14}C]2-deoxy-D-glucose into the brain was also decreased by the administration of cysteamine. Cysteamine dose dependently increased gastric acid secretion and induced ulcers in the gastroduodenum. The increased in acid secretion and the decrease of the value of glucose uptake into the brain had related time courses. Cysteamine significantly increased the level of serum insulin and induced hypoglycemia. These effects of cysteamine were completely blocked by pretreatment with streptozotocin. The hypersecretion of gastric acid and gastroduodenal ulcerations were also significantly inhibited by streptozotocin pretreatment. Infusion of glucose also inhibited the cysteamine-induced gastric acid secretion. These results suggest that cysteamine enhances the gastric acid secretion induced by the hypoglycemia and by the decrease in glucose uptake by the brain.

Cysteamine; Ulcer; Insulin; Gastric acid secretion; Glucose uptake; Brain; (Rat)

1. Introduction

Cysteamine causes perforating duodenal ulcers in rats (Selye and Szabo, 1973). Factors involved in the ulceration include hypersecretion of acid (Groves et al., 1974; Fujii and Ishii, 1975; Ishii et al., 1976; Tamaki et al., 1978; Szabo et al., 1979), delayed gastric emptying (Lenard et al., 1977; Poulsen et al., 1982), reduction in duodenal regional blood flow (Kurebayashi et al., 1985) and reduced secretory activity of Brunner's gland in the duodenum (Kirkegaard et al., 1981). The hypersecretion of gastric acid is closely linked to induction of ulcers by cysteamine since anti-acid secretory agents, anticholinergics and H_2 antagonists prevent ulceration (Fujii and Ishii, 1975; Ishii et al., 1976; Szabo et al., 1979). With regard to the mechanism of the hypersecretion, an increase in neuronal activity of the vagus nerve has been proposed, based on evidence that this hypersecretion is inhibited by cervical vagotomy and by pretreatment with hexamethonium and atropine (Fujii and Ishii, 1975; Ishii et al., 1976).

The mechanism of the vagal-mediated gastric acid secretion induced by insulin and 2-deoxy-D-glucose has not yet been clarified (Bachach, 1953; Eisenberg et al., 1966). It was suggested that the vagal-mediated gastric acid secretion is controlled by hypothalamic neuronal activity (Porter et al., 1953; Ridley, 1965; Misher and Brooks, 1966; Davis and Weiner, 1969; Mason and Neisen, 1969) and that the most important factor regulating the activity is brain glucose levels (Brooks, 1967; Himsworth, 1968; Colin-Jones and Himsworth, 1969; Oomura et al., 1974; Oomura, 1976; Hirano and Nijima, 1980). We reported that the increase in gastric acid output in rats is associated with a decrease in glucose uptake by the brain, under conditions of restraint and water-immersion stress and insulin-induced hypoglycemia (Arai et al., 1987). We now report on the effect of cysteamine on blood glucose levels, glucose uptake into the brain and gastric acid output of rats.

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2. Materials and methods

2.1. Animals

Male Wistar strain rats (180–250 g) were deprived of solid food for 18 h but were allowed free access to tap water for drinking. Each group included 6–12 rats.

2.2. Measurement of blood glucose levels

The rats were anesthetized with ether, restrained in a supine position and all surgical regions were treated thoroughly with lidocaine (Pfizer). Under ether anesthesia, the superior cervical region was incised along the midline, and the right carotid artery and right jugular vein were exposed. About 50 μl of the blood from each vessel were collected into a disposable syringe (Terumo, SS-01T2613S). The blood glucose level was determined using glucose oxidase and Dextrostics and a Dextrometer II (Mails-Sankyo), hourly for 6 h following the administration of cysteamine (300 mg/kg s.c.) or insulin (3 U/kg s.c.). This dose of insulin was sufficient to increase gastric acid secretion in rats as reported previously (Arai et al., 1985). The value obtained by subtracting jugular venous blood glucose from arterial blood glucose was considered to be the amount of glucose uptake of the brain.

2.3. Measurement of gastric acid secretion

Two experimental methods were used in this study. (1) Cysteamine (300 mg/kg s.c.)- and insulin (3 U/kg s.c.)-stimulated gastric acid secretion was examined in conscious rats with an acute gastric fistula. (2) Gastric acid secretion stimulated by cysteamine (100 mg/kg s.c.), methacholine (0.3 mg/kg s.c.), gastrin (0.3 mg/kg s.c.) and histamine (3 mg/kg s.c.) was examined in urethane (1.25 g/kg s.c.)-anesthetized rats with an acute gastric fistula. The responses to secretagogues including cysteamine were greater in anesthetized than in conscious rats. We then used the different doses of cysteamine in each experimental protocol.

An acute gastric fistula (KN-365 R-1C, Natume) was prepared. Under ether anesthesia, the pylorus was ligated and a cannula was inserted into the forestomach to collect the gastric juice. Gastric juice was collected into a test tube every 30 or 60 min for 3.5 or 6 h by washing out with 10 ml of saline warmed to 37 °C. The total acid output in each sample was determined by titration with 0.01 N NaOH, using phenolphthalein as indicator.

2.4. Measurement of [^{14}C]2-deoxy-D-glucose uptake into the brain

One hour after cysteamine (300 mg/kg s.c.) administration, 0.5 μCi of [^{14}C]2-deoxy-D-glucose ([^{14}C]2-DG) (New England Nuclear, S.A.: 51.1 mCi/mol) in 0.1 ml physiological saline was given i.v. and the animals were decapitated 5 min later. Arterial blood was collected just before decapitation, to determine the glucose levels. The brain was rapidly removed and separated into seven parts as described by Glowinski and Iversen (1966). The radioactivity of [^{14}C]2-DG uptake into these regions was measured in a liquid scintillation counter (Packaed model, 4430) after solubilization with NCS (Amersham Co., Inc.).

2.5. Measurement of serum insulin and glucagon concentration

About 3 ml of blood was collected from each decapitated rat. The serum obtained by centrifugation was stored at −20 °C until assayed. Serum insulin and glucagon were radioimmunoassayed with a Rat Insulin kit (Dainabot) and a Glucagon kit (Daiichi), respectively. All samples were tested in duplicate. The sensitivity of the insulin and the glucagon assay was 10 μU/ml and 15 pg/ml serum, respectively.

2.6. Pretreatment with streptozotocin

Streptozotocin, in a dose of 30 mg/kg i.v., was used in this study since this dose partially destroys pancreatic β cells and significantly increases the blood glucose level. This dose of streptozotocin had no influence on blood pressure and heart rate as determined in a preliminary study. Streptozotocin was given i.v. 24 h before the administration of cysteamine.

2.7. Lesion induction

We examined the effect of streptozotocin on the development of cysteamine-induced gastroduodenal lesions in rats. Cysteamine, 300 mg/kg, was given s.c. 24 h after the administration of streptozotocin. The animals were killed 18 h later, the stomach and duodenum were removed, disfended with 1% formalin solution and placed into the same solution for 10 min. These organs were then opened by cutting along the greater curvature and the lesions were examined. The ulcer index was determined from the area of each lesion, using stereoscopic microscopy.

2.8. Drugs

The drugs used were cysteamine (Nakarai), methacholine chloride (Nakarai), streptozotocin (Sigma), histamine dihydrochloride (Wako), urethane (Wako), glucose (Wako) and gastrin (Gastopcin, Nihon-kayaku). All the drugs were dissolved in saline solution and given in a volume of 2 ml/kg body weight, except streptozotocin that was given in a volume of 1 ml/kg body weight.

2.9. Statistical analysis

All results are expressed as the means ± S.E. The differences in values among the various groups were analyzed using a one-way analysis of variance and

Dunnett's test. P values less than 0.05 are considered to have statistical significance.

3. Results

3.1. Blood glucose level and acid secretion

Cysteamine (100–300 mg/kg s.c.) significantly decreased arterial blood glucose level, in a dose-dependent manner 1 h after its administration, but had no effects on the jugular venous blood glucose level (table 1). The value of glucose uptake into the brain was also significantly decreased, in a dose dependent manner, by the administration of cysteamine.

As shown in table 2, cysteamine (300 mg/kg s.c.) significantly decreased only arterial blood glucose level, which reached a plateau after 1 and 4 h. The control value for glucose uptake into the brain 45.3 ± 6.7 mg/dl was significantly decreased and reached a minimum 4.1 ± 1.2 mg/dl 1 h after the cysteamine administration. The value of glucose uptake into the brain remained below the basal level during the experiment. Cysteamine (300 mg/kg s.c.) significantly increased gastric acid secretion, with a peak 4 h after its administration. The increase in acid output induced by cysteamine was almost inversely related to the decrease of the value of glucose uptake into the brain except 1 h after the cysteamine administration. The increase in the acid output was about 1 h later than the decrease of the values of glucose uptake into the brain.

Insulin (3 U/kg s.c.) significantly decreased both arterial blood glucose and jugular venous blood glucose levels (table 2, lower part). The value of glucose uptake into the brain in the insulin-treated group was significantly decreased and reached a minimum (4.8 ± 1.0 mg/dl) 2 h after the administration. Thereafter, the value increased gradually but the value obtained 6 h after the administration was about one third of the control. The time course of the changes in the acid output induced by insulin was in fairly good inverse relation to the decrease of the value of glucose uptake into the brain (table 2).

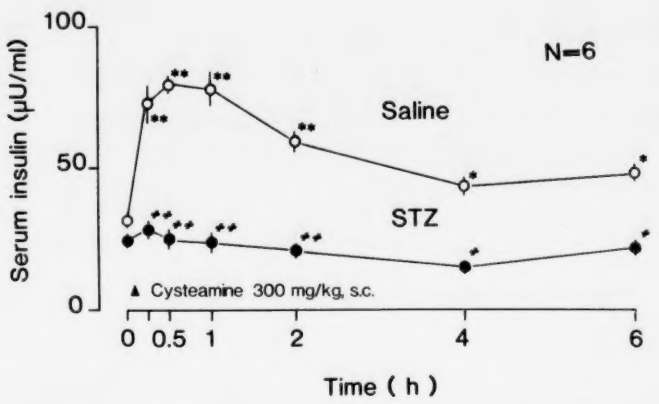

Fig. 1. Effect of streptozotocin on serum insulin concentration in cysteamine-treated rats. (○) Saline was given 1 ml/kg i.v. 24 h before cysteamine administration; (●) streptozotocin was given 30 mg/kg i.v. 24 h before cysteamine administration. Each value represents the mean ± S.E. * $P < 0.05$ and ** $P < 0.01$ compared with the 0 time value for each group. # $P < 0.05$ and ## $P < 0.01$ compared with the value for the saline group at each time.

3.2. [^{14}C]2-DG uptake into the brain

A significant decrease of [^{14}C]2-DG uptake into the brain was observed 1 h after the administration of cysteamine (300 mg/kg s.c.). There was no regional difference in the degree of decrease in [^{14}C]2-DG uptake into the brain in rats given cysteamine (table 3).

3.3. Serum insulin concentration

The control serum insulin concentration in the normal rats was 32.1 ± 3.1 μU/ml. Cysteamine (300 mg/kg s.c.) significantly increased the serum insulin level with a peak (79.9 ± 2.3 μU/ml) 30 min after its administration (fig. 1, open circle). Streptozotocin did not have any effect on serum insulin levels but clearly prevented

TABLE 1

Effects of cysteamine and streptozotocin on blood glucose levels in rats.

ABG, arterial blood glucose level; VBG, jugular venous blood glucose level; ABG-VBG, glucose uptake into the brain. STZ, streptozotocin was given 30 mg/kg i.v. 24 h before cysteamine. Blood samples were collected 1 h after cysteamine administration. Each value represents the mean ± S.E.

Drug	Dose (mg/kg)	N	ABG (mg/dl)	VBG (mg/dl)	ABG-VBG (mg/dl)
Control	–	8	105.4 ± 5.6	59.9 ± 4.2	45.5 ± 3.4
Cysteamine	30	8	106.4 ± 7.8	66.6 ± 6.0	39.9 ± 10.9
Cysteamine	100	8	83.8 ± 6.9 [a]	63.0 ± 3.3	20.8 ± 6.6 [a]
Cysteamine	300	8	65.3 ± 3.1 [b]	55.0 ± 4.3	10.3 ± 5.4 [b]
STZ	30	8	149.9 ± 9.7 [b]	95.1 ± 11.0 [b]	54.6 ± 13.9
Cysteamine + STZ	300 + 30	8	214.3 ± 40.8 [d]	156.7 ± 32.1 [a,d]	57.7 ± 17.7 [c]

[a] $P < 0.05$ and [b] $P < 0.01$ compared with the control. [c] $P < 0.05$ and [d] $P < 0.01$ compared with the value for the group given cysteamine 300 mg/kg.

TABLE 2

Effects of cysteamine and insulin on blood glucose levels and gastric acid secretion in rats.

ABG, arterial blood glucose level; VBG, jugular venous blood glucose level; ABG-VBG, glucose uptake into the brain. Each value represents the mean ± S.E. for six animals.

Drug	Time after (treatment (h)	ABG (mg/dl)	VBG (mg/dl)	ABG-VBG (mg/dl)	Acid output (μEq/h)
Cysteamine	0	101.4 ± 7.5	56.1 ± 4.4	45.3 ± 6.7	24.1 ± 11.0
(300 mg/kg s.c.)	1	52.0 ± 4.4 [b]	50.8 ± 5.0	4.1 ± 1.2 [b]	18.6 ± 4.1
	2	52.8 ± 3.7 [b]	48.0 ± 5.2	5.3 ± 4.5 [b]	43.6 ± 4.6 [b]
	4	40.5 ± 2.0 [b]	37.5 ± 4.6 [a]	4.4 ± 3.0 [b]	59.2 ± 2.1 [b]
	6	62.5 ± 1.6 [b]	49.8 ± 3.0	12.7 ± 2.5 [b]	38.4 ± 9.4
Insulin	0	119.4 ± 7.2	68.9 ± 3.3	50.5 ± 6.4	24.0 ± 4.1
(3 U/kg s.c.)	1	39.5 ± 2.4 [b]	32.3 ± 0.8 [b]	7.2 ± 2.6 [b]	45.3 ± 9.1 [a]
	2	32.7 ± 1.6 [b]	27.8 ± 1.9 [b]	4.8 ± 1.0 [b]	70.7 ± 5.1 [b]
	4	41.3 ± 3.2 [b]	26.5 ± 2.2 [b]	14.8 ± 2.3 [b]	30.7 ± 10.7
	6	56.3 ± 4.1 [b]	39.2 ± 2.9 [b]	17.2 ± 1.4 [b]	22.7 ± 2.0

[a] $P < 0.05$ and [b] $P < 0.01$ compared with each parameter value at 0 time.

TABLE 3

Effect of cysteamine on [^{14}C]2-deoxy-D-glucose uptake into various brain regions in rats.

Each value represents the mean ± S.E. for six animals.

Brain region	[^{14}C]2-Deoxy-D-glucose (pmol/g tissue per min)	
	Control	Cysteamine) (300 mg/kg)
Cerebellum	36.5 ± 4.1	14.8 ± 0.5 [b]
Medulla oblongata	27.0 ± 4.2	14.7 ± 0.8 [a]
Striatum	25.5 ± 2.2	16.2 ± 0.7 [b]
Hypothalamus	40.0 ± 6.3	17.9 ± 2.3 [b]
Midbrain	31.6 ± 3.2	15.4 ± 0.6 [b]
Hippocampus	30.1 ± 5.6	16.1 ± 1.6 [a]
Cortex	36.6 ± 2.1	17.5 ± 1.5 [b]

[a] $P < 0.05$ and [b] $P < 0.01$ compared with each regional value for the control.

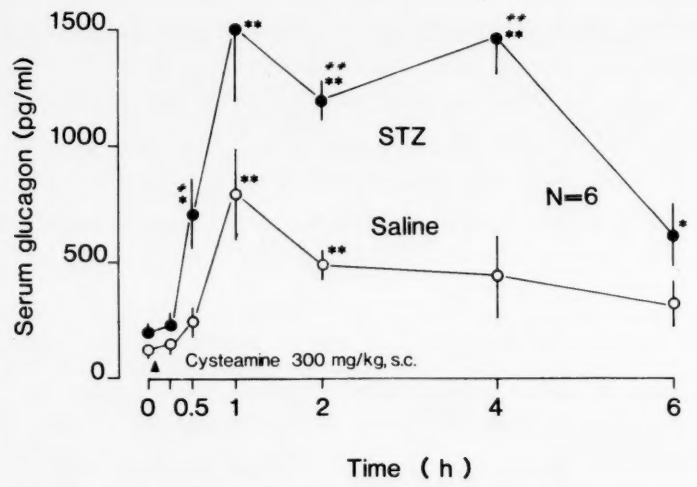

Fig. 2. Effect of streptozotocin on serum glucagon concentration in cysteamine-treated rats. (○) Saline was given 1 ml/kg i.v. 24 h before cysteamine; (●) streptozotocin was given 30 mg/kg i.v. 24 h before cysteamine. Each value represents the mean ± S.E. * $P < 0.05$ and ** $P < 0.01$ compared with the value for each group at 0 time. # $P < 0.05$ and ## $P < 0.01$ compared with value for the saline group at each time.

the increase of serum insulin induced by cysteamine (fig. 1, closed circle).

3.4. Serum glucagon concentration

The basal serum glucagon concentration in the normal rats was 134.0 ± 18.4 pg/ml. Cysteamine (300 mg/kg s.c.) significantly increased the serum glucagon level with a peak (820.0 ± 197.9 pg/ml) 1 h after its administration (fig. 2, open circle). Though streptozotocin did not have any effect on the serum glucagon level it enhanced the cysteamine-induced increase in serum glucagon (fig. 2, closed circle).

3.5. Effect of streptozotocin on the blood glucose levels

Streptozotocin significantly increased arterial blood glucose level and the jugular venous blood glucose level to a similar degree and had no effect on the value of glucose uptake into the brain. The decrease in the

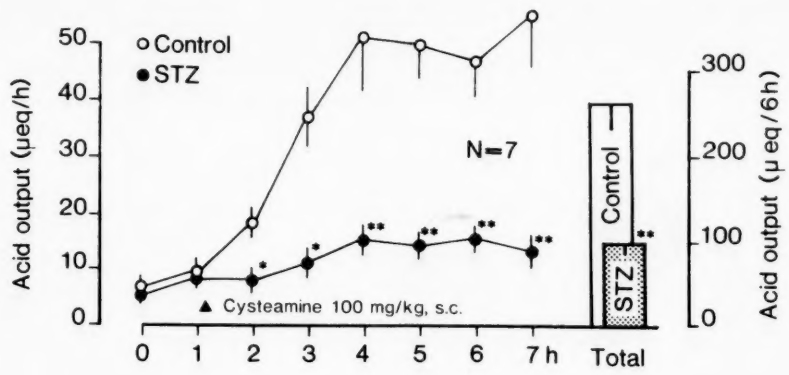

Fig. 3. Effect of streptozotocin on cysteamine-induced gastric acid secretion in rats. (○) Saline was given 1 ml/kg i.v. 24 h before cysteamine; (●) streptozotocin was given 30 mg/kg i.v. 24 h before cysteamine. Each value represents the mean ± S.E. * $P < 0.05$ and ** $P < 0.01$ compared with each value for the saline group.

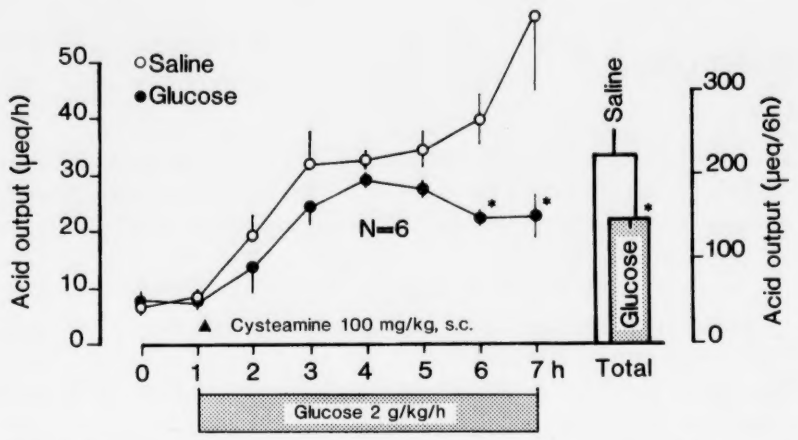

Fig. 4. Effect of glucose on cysteamine-induced gastric acid secretion in rats. (○) Saline was 10 ml/mg per h; (●) glucose 2 g/kg per h. Each value represents the mean ± S.E. * $P < 0.05$ compared with the saline group.

value of glucose uptake into the brain induced by cysteamine was completely blocked by streptozotocin (table 1).

3.6. Effect of streptozotocin on gastric acid output

As shown in fig. 3, cysteamine (100 mg/kg s.c.) increased the gastric acid output time dependently and with a steady state reached 4 h after the administration. Streptozotocin markedly inhibited the cysteamine-stimulated gastric acid output but did not have any effect on methacholine-, gastrin- and histamine-stimulated gastric acid output (data not shown).

3.7. Effect of glucose on cysteamine-induced gastric acid output

Cysteamine (100 mg/kg s.c.) increased the gastric acid output time dependently. Infusion of glucose (2 g/kg per h i.v.) prevented the hypoglycemic effect of cysteamine in arterial blood (data not shown). Though the glucose infusion did not inhibit the early phase of the cysteamine-induced gastric acid secretion, it significantly inhibited the later phase (fig. 4). The total acid output was also significantly inhibited by the glucose infusion.

TABLE 4

Effect of streptozotocin on cysteamine-induced ulceration in rats.

STZ, streptozotocin (30 mg/kg i.v.) was given i.v. 24 h before cysteamine (300 mg/kg s.c.). Each value represents the mean ± S.E.

Drug	Dose (mg/kg)	N	Ulcer area (mm^2)	
			Stomach	Duodenum
Cysteamine	–	12	1.31 ± 0.41	9.35 ± 1.56
Cysteamine + STZ	30	12	0.09 ± 0.04 [b]	3.13 ± 1.71 [a]

[a] $P < 0.05$ and [b] $P < 0.01$ compared with the value for the cysteamine alone group.

3.8. Gastroduodenal lesions

Cysteamine produced severe ulceration in the duodenum with a slight ulceration in the stomach. Pretreatment with streptozotocin significantly prevented these cysteamine-induced ulcerations (table 4).

4. Discussion

Blood samples of arterial blood and the jugular venous blood were obtained from the carotid artery and jugular vein, respectively. The margin in concentrations between arterial blood glucose level and the jugular venous blood glucose level were considered to be the amount of glucose consumed in the brain. There was a close relationship between the value of glucose uptake into the brain, but not the jugular venous blood glucose level, and [^{14}C]2-DG uptake into the brain in restrained and water-immersion-stressed and insulin-treated rats (Arai et al., 1987). Under these conditions, the glucose uptake into the brain was inversely related to the increase in gastric acid secretion. Cysteamine-induced acid secretion was also related to the decrease of the value of glucose uptake into the brain but not to the arterial blood glucose level and the jugular venous blood glucose level. Therefore, the cysteamine-induced hypersecretion of gastric acid might be regulated by glucose uptake into the brain similar to the case of restraint and water-immersion stress and insulin administration, as reported earlier (Arai et al., 1987).

After 15 min cysteamine significantly increased the serum insulin level which reached a peak 30 min after administration. Thereafter, the serum insulin concentration decreased gradually, but remained significantly higher than at 0 time (fig. 1). The result suggests that cysteamine stimulates the pancreatic β cells to increase the insulin levels in serum. Moreover, streptozotocin is known to cause selective destruction of pancreatic β cells and induce a hypoinsulinemic diabetes mellitus (Nathan et al., 1963). The cysteamine-induced hyperinsulinosis was completely inhibited by streptozotocin pretreatment. These results suggest that the hypoglycemia induced by cysteamine is due to hyperinsulinosis caused by cysteamine.

The cysteamine-stimulated gastric acid output was significantly diminished by streptozotocin pretreatment, but stimulation of gastric acid output by the peripheral site-stimulating secretagogues, methacholine, gastrin and histamine was not influenced by streptozotocin. These results suggest that the site of action of streptozotocin is the periphery and that the effect is exerted via the central nervous system.

Pretreatment with streptozotocin inhibited by cysteamine-induced hypoglycemia and the decrease of glucose uptake by the brain. Cysteamine stimulated glucagon release. This means that cysteamine simultaneously stimulates pancreatic α and β cells to increase the serum glucagon and insulin levels. Streptozotocin enhanced the cysteamine-increased serum glucagon and increased the blood glucose level (fig. 2). Though the reason for the former effect is not clear the increase of blood glucose may be due to the increase of serum glucagon.

Glucose (2 g/kg per h) inhibited cysteamine-induced gastric acid secretion. This also suggests that glucose acts as an inhibitory factor for acid secretion. The inhibitory effect of glucose on the cysteamine-induced gastric acid secretion was not as clearly demonstrated as that of streptozotocin. This may be result of the effect of glucose infusion on insulin secretion. The glucose infusion may stimulate the release of insulin from the pancreatic cells, and this insulin would then decrease the blood glucose level, and subsequently make it difficult to observe a clear effect of glucose infusion on acid secretion.

These results suggest that acid secretion caused by cysteamine and mediated by the decrease of glucose uptake into the central nervous system is, like the gastric acid secretion induced by insulin and restraint and water-immersion stress, regulated by brain glucose levels (Arai et al., 1987). Restraint and water-immersion stress induce a decrease of glucose uptake by the brain and may be the result of hyperglycemia in which a significant decrease in glucose uptake is caused by a marked increase in the jugular venous blood glucose level. The results thus also suggest that stressful conditions inhibit glucose consumption by the brain as indicated by the increase in the jugular venous blood glucose level. All these observations are taken to mean that the cysteamine-induced decrease in the value of glucose uptake into the brain may be the combined effects of insulin-induced hypoglycemia and a stress-induced decrease in brain glucose consumption.

Cysteamine-induced gastroduodenal ulceration was also significantly inhibited by streptozotocin, perhaps due to the inhibitory effect of streptozotocin on gastric acid secretion.

In conclusion, cysteamine-induced hypersecretion of gastric acid may be related to the decrease of glucose uptake by the brain, as related to increases in endogenous insulin secretion.

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References

Arai, I., H. Hirose, M. Muramatsu, S. Okuyama and H. Aihara, 1985, Possible involvement of non-steroidal anti-inflammatory drugs in vagal-mediated gastric acid secretion in rats, Jap. J. Pharmacol. 37, 91.

Arai, I., H. Hirose, M. Muramatsu and H. Aihara, 1987, Effects of restrain and water-immersion stress and insulin on gastric acid secretion in rats, Physiol. Behav. 40, 357.

Bachach, W.H. 1953, Action of insulin hypoglycemia on motor and secretory function of the digestive tract, Physiol. Rev. 33, 566.

Brooks, F.P., 1967, Central neural control of acid secretion, in: Handbook of Physiology, Section 6: Alimentary Canal, Vol. II Section, Chapter 45 (American Physiological Society, Washington, DC) p. 805.

Colin-Jones, D.G. and R.L. Himsworth, 1969, The secretion of gastric acid in response to a lack of metabolizable glucose, J. Physiol. 202, 97.

Davis, R.A. and S. Weiner, 1969, Anterior hypothalamic regulation of gastric secretion, Surg. Gynecol. Obstet. 128, 1007.

Eisenberg, M.M., G.S. Emas and M.I. Grossman, 1966, Comparison of the effect of 2-deoxy-D-glucose and insulin on gastric acid secretion in dogs, Surgery 60, 111.

Fujii, Y. and Y. Ishii, 1975, Influence of various factors and drugs on cysteamine-induced duodenal ulcers in the rat, Jap. J. Pharmacol. 25, 663.

Glowinski, J. and L.L. Iversen, 1966, Regional studies of catecholamines in the rat brain – I, J. Neurochem. 13, 655.

Groves, W.G., J.H. Scheossen and F.D. Mead, 1974, Acid hypersecretion and duodenal ulcers produced by cysteamine in rats, Res. Commun. Chem. Pathol. Pharmacol. 9, 523.

Himsworth, R.L., 1968, Interference with the metabolism of glucose by a non-metabolizable hexose (3-methyl glucose), J. Physiol. 198, 467.

Hirano, T. and A. Niijima, 1980, Effects of 2-deoxy-D-glucose, glucose and insulin on efferent activity in gastric vagus nerve, Experientia 36, 1197.

Ishii, Y., Y. Fujii and M. Homma, 1976, Gastric acid stimulating action of cysteamine in the rat, Eur. J. Pharmacol. 36, 331.

Kirkegaard, P., S.S. Poulsen, C. Halse, F.B. Loud, O.P. Skov and J. Christiansen, 1981, The effect of cysteamine on the Brunner gland secretion in the rat, Scand. J. Gastroenterol. 16, 93.

Kurebayashi, Y., M. Asano, T. Hashizume and A. Akashi, 1985, Changes in duodenal mucosal blood flow and mucus glycoprotein content during cysteamine-induced duodenal ulceration in rats, Arch. Int. Pharmacodyn. 276, 152.

Lenard, M., L.M. Lichtenberger, S. Szabo and E.S. Reynolds, 1977, Gastric emptying in the rat is inhibited by the duodenal ulcerogens cysteamine and propionitrile, Gastroenterology 73, 1072.

Mason, G.R. and T.S. Neisen, 1969, Gastric secretory and motor responses to anterior hypothalamic stimulation, Am. J. Physiol. 217, 1771.

Misher, A. and F.P. Brooks, 1966, Electrical stimulation of hypothalamus and gastric secretion in the albino rat, Am. J. Physiol. 211, 403.

Nathan, R., L.R. Morris and V.N. Moreshwar, 1963, Studies on the diabetogenic action of streptozotocin (NSC-37917), Cancer Chemother. Rep. 29, 91.

Oomura, Y., 1976, Significance of glucose, insulin and free fatty acid on the hypothalamic feeding and satiety neurons, in: Hunger: Basic Mechanism and Clinical Implications, eds. D. Noven, W. Wyrwicka and G.A. Brag (Raven Press, New York) p. 145.

Oomura, Y., H. Ooyama, M. Sugimori, T. Nakamura and Y. Yamada, 1974, Glucose inhibition of the glucose sensitive neurons in the rat lateral hypothalamus, Nature 247, 284.

Porter, R.W., H.J. Movius and J.D. French, 1953, Hypothalamic

influences on hydrochloric acid secretion of the stomach, Surgery 33, 875.

Poulsen, S.S., P. Kirkegaard, O.P. Skov and J. Christiansen, 1981, Brunner's glands of the rat during cysteamine ulceration, Scand. J. Gastroenterol. 16, 93.

Poulsen, S.S., P. Kirkegaard, O.P. Skov, K.K. Jensen and J. Christansen, 1982, Role of delayed gastric emptying in the pathogenesis of cysteamine-induced duodenal ulcer in the rat, Scand. J. Gastroenterol. 17, 325.

Ridley, P.T. and F.P. Brooks, 1965, Alterations in gastric secretion following hypothalamic lesions producing hyperphagia, Am. J. Physiol. 209, 319.

Selye, H. and S. Szabo, 1973, An experimental model for the production of perforating duodenal ulcers by cysteamine in the rat, Nature (London) 244, 458.

Szabo, S., L.R. Haith and E.S. Reynolds, 1979, Pathogenesis of duodenal ulceration produced by cysteamine or propionitrile. Influences of vagotomy, sympathectomy, histamine depletion, H-2 receptor antagonists and hormones, Dig. Dis. Sci. 24, 471.

Tamaki, H., Y. Onoda and T. Kashida, 1978, Gastric secretion and duodenal ulcer formation induced by cysteamine in rats, Jap. J. Pharmacol. 28, 647.

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Similar effects of treatment with desipramine and electroconvulsive shock on 5-hydroxytryptamine$_{1A}$ receptors in rat brain

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The effect of chronic and acute treatment with desipramine (DMI) and electroconvulsive shock (ECS) on 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptors was determined in the cortex and the hippocampus brain regions of rats. We observed that chronic treatment with both DMI and ECS significantly decreased 5-HT$_{1A}$ receptors, as determined by [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]8-OH-DPAT) binding, in the cortex but not in the hippocampus. Acute treatment with DMI or ECS did not significantly alter the 5-HT$_{1A}$ receptors in the cortex. Neither chronic nor acute treatment influenced K_D of [^{3}H]8-OH-DPAT binding in the cortex or hippocampus. These results thus suggest that in contrast to the effects on 5-HT$_2$ receptors, tricyclics as well as ECS produce similar effects on 5-HT$_{1A}$ receptors, suggesting that this site may represent a common site of action for antidepressant treatment.

5-HT$_{1A}$ receptors; 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin); Desipramine; Electroconvulsive shock; Brain (rat)

1. Introduction

Several lines of evidence suggest that 5-hydroxytryptamine (5-HT) receptor subtypes may be involved in the pathophysiology of depression and in mechanisms of action of antidepressant drugs (Willner, 1985; Meltzer and Lowy, 1987; Pandey et al., 1990; Lesch et al., 1990; Peroutka and Snyder, 1980). Chronic treatment with antidepressant drugs causes down regulation of 5-HT$_2$ receptors and a decrease in 5-HT$_2$ receptor-mediated inositol phosphate formation in rat brain (Peroutka and Snyder, 1980; Kendall and Nahorski, 1985). However, repeated administration of electroconvulsive shock (ECS) causes up regulation of 5-HT$_2$ receptors in rat brain (Kellar et al., 1981). Because of these opposite effects of antidepressant drugs and ECS, the association of 5-HT$_2$ receptors with antidepressant effects is questionable. On the other hand, repeated administration of both antidepressant drugs and ECS produces similar effects on 5-HT$_{1A}$ receptor-mediated physiological and behavioral responses in rats and mice (Green, 1987; Wozniak et al., 1988; Goodwin et al., 1985). For example, repeated administration of antidepressant drugs as well as ECS attenuates both hypothermic response and the serotonin syndrome produced by the administration of 5-HT$_{1A}$ receptor-specific agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Green, 1987). As opposed to the effects on 5-HT$_2$ receptors, similar effects of antidepressant drugs and ECS on 5-HT$_{1A}$ receptor-mediated response thus suggest that 5-HT$_{1A}$ receptors may be involved in the mechanism of action of antidepressant drugs.

Biochemical correlates of the above physiological and behavioral findings are less clear and have not been systematically studied. It has been shown by Newman and Lerer (1988) that chronic ECS and desipramine (DMI) treatment causes a decrease in 5-HT-induced inhibition of forskolin-stimulated adenylate cyclase activity in the hippocampus brain region of rat, thus suggesting down regulation of 5-HT$_{1A}$ receptors. Mizuta and Segawa (1988) reported that chronic treatment with imipramine caused down-regulation of 5-HT$_{1A}$ receptors, as measured by [^{3}H]8-OH-DPAT binding in rat cortex and hippocampus. However, Newman et al. (1990) reported no change in 5-HT$_{1A}$ receptor binding in rat hippocampus after chronic treatment with DMI. Whereas effects of DMI on [^{3}H]8-OH-DPAT binding sites in hippocampus are thus controversial, the effect of ECS on 5-HT$_{1A}$ receptors has not yet been investigated. To further examine the role of 5-HT$_{1A}$ receptors in the mechanism of action of antidepressant drugs, we studied the effect of repeated and single administration of DMI and ECS on 5-HT$_{1A}$

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binding sites using [^{3}H]8-OH-DPAT as the ligand in rat brain. Since 5-HT_{1A} receptors are located pre- and postsynaptically in cortex (Hall et al., 1985) but postsynaptically in hippocampus (Hamon et al., 1984; Gozlan et al., 1983), we examined the effect of acute and chronic administration of DMI and ECS on [^{3}H]8-OH-DPAT binding in both cerebral cortex and hippocampus brain regions of rat.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (200–250 g) were used throughout the study. The rats were housed in a temperature- and light-controlled room and had free access to food and water.

2.2. Treatment with ECS or DMI

The rats received a single ECS (acute) or ECS once daily for 14 days (chronic). Current (75 mA for 0.2 s) was delivered through earclip electrodes. Each shock elicited tonic-clonic seizures that lasted approximately 40 s. Control rats (sham) were handled in the same way, but no current was delivered.

Another group of rats was administered with intraperitoneal (i.p.) injection of DMI (10 mg/kg) once daily for 14 days (chronic DMI) or 1 day (acute DMI). Control rats received injections of the same volume of saline. In the acute and the chronic studies we used different batches of rats. The ages of the rats in the two studies were different. Rats were killed by decapitation 24 h after the last administration of DMI or ECS for chronic studies and after 1 h of single administration of DMI or ECS for acute studies. The brains were removed and cortices and hippocampi were dissected out and stored at −70°C until assayed.

2.3. Determination of the [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors in rat cortical and hippocampal membranes

Cortices and hippocampi were homogenized in 10 volumes of 0.32 M sucrose using a polytron, with a setting at 9 for 30 s, and centrifuged at $1000 \times g$ for 10 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at $70\,000 \times g$ for 15 min and the pellet thus obtained was suspended in 10 volumes of Tris buffer (50 mM, pH 7.5). The suspension was incubated at 37°C for 15 min and was centrifuged again at $70\,000 \times g$ for 15 min. The final pellet was suspended in incubation buffer (50 mM Tris, 4 mM $CaCl_2$, 0.1% ascorbic acid, pH 7.7). Binding of [^{3}H]8-OH-DPAT was carried out in triplicate by incubation of a 500-μl aliquot of membrane suspension with [^{3}H]8-OH-DPAT at five to six concentrations (between 0.5–10 nM) in incubation buffer with or without 10 μM 5-HT in a total volume of 1.0 ml at 37°C for 30 min. [^{3}H]8-OH-DPAT bound to the membrane was separated by filtration through a Whatman (GF/B) filter and washed four times with 5.0 ml cold buffer (50 mM Tris, pH = 7.5) using a Brandel cell harvester. The filters were placed in vials and scintillation cocktail was added and counted in a scintillation counter. Specific binding is defined as the difference between total binding and the binding observed in the presence of 10 μM 5-HT and ranged from 90-70% depending upon the concentration of [^{3}H]8-OH-DPAT. The maximum number of binding sites (B_{max}) and apparent dissociation constant (K_D) were computed by Scatchard analysis using the EBDA program (McPherson, 1985). Protein was measured by the method of Lowry et al. (1951).

2.4. Statistical methods

Student's *t*-test was used to compare means of B_{max} and K_D of two groups, e.g. sham vs. ECS and saline vs. DMI. A value of $P < 0.05$ was considered to be significant.

3. Results

The binding characteristics of [^{3}H]8-OH-DPAT binding to cortical and hippocampal membranes were similar to those reported by other investigators (Hall et al., 1985; Gozlan et al., 1983; Newman et al., 1990). The saturation isotherm and Scatchard plot (inset) of [^{3}H]8-OH-DPAT binding to hippocampal and cortical membranes are shown in fig. 1. As shown in fig. 1 specific binding appeared to be saturable, and the Scatchard plot from a typical saturation experiment indicated a single class of high-affinity binding sites in cortex and hippocampus. The B_{max} of [^{3}H]8-OH-DPAT binding was higher in hippocampus, in comparison to cortex, whereas similar K_D values of [^{3}H]8-OH-DPAT binding were found in both cortical and hippocampal membranes. Similar regional distribution patterns of [^{3}H]8-OH-DPAT binding sites have also been reported by other investigators (Hall et al., 1985; Gozlan et al., 1983).

3.1. Effect of acute and chronic DMI and ECS on 5-HT_{1A} receptors in rat cortex

A single ECS administration or a single injection of DMI did not significantly alter either the number (B_{max}) or affinity (K_D) of [^{3}H]8-OH-DPAT binding sites in rat cortex. However, chronic treatment with DMI (t value

TABLE 1

Effect of treatment with DMI or ECS on [^{3}H]8-OH-DPAT binding in cortex.

Values are means ± S.E.M., except values within parentheses, which indicate the number of experiments. Significantly different from controls: [a] $P < 0.03$; [b] $P < 0.003$.

Groups	Chronic treatment		Acute treatment	
	B_{max} (fmol/mg protein)	K_D (nM)	B_{max} (fmol/mg protein)	K_D (nM)
Saline	(12) 129 ± 8.2	2.6 ± 0.49	(5) 81 ± 5.9	1.5 + 0.15
DMI	(12) 102 ± 7.9 [a]	2.3 ± 0.29	(5) 77 ± 5.8	1.5 + 0.13
Sham	(8) 132 ± 5.5	1.8 ± 0.21	(5) 93 ± 9.4	2.1 + 0.33
ECS	(8) 107 ± 4.5 [b]	2.1 ± 0.21	(5) 83 ± 6.8	2.1 + 0.62

TABLE 2

Effect of chronic treatment with DMI or ECS on [^{3}H]8-OH-DPAT binding in hippocampus.

Values are means ± S.E.M. from the number of experiments, which are shown within parentheses.

Groups	B_{max} (fmol/mg protein)	K_D (nM)
Saline (10)	235 ± 21.6	2.7 ± 0.36
DMI (10)	207 ± 22.8	2.6 ± 0.25
Sham (8)	259 ± 16.9	2.1 ± 0.31
ECS (8)	286 ± 18.4	1.9 ± 0.26

2.31, df = 22, $P < 0.03$) or ECS (t value = 3.51, df = 14, $P < 0.003$) significantly decreased the B_{max} of [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors in rat cortex without altering the affinity (K_D) of the [^{3}H]8-OH-DPAT binding sites. These results are shown in table 1.

3.2. Effect of chronic DMI and ECS on 5-HT_{1A} receptors in rat hippocampus

We also studied the effect of DMI and ECS on 5-HT_{1A} receptors in rat hippocampus. We observed

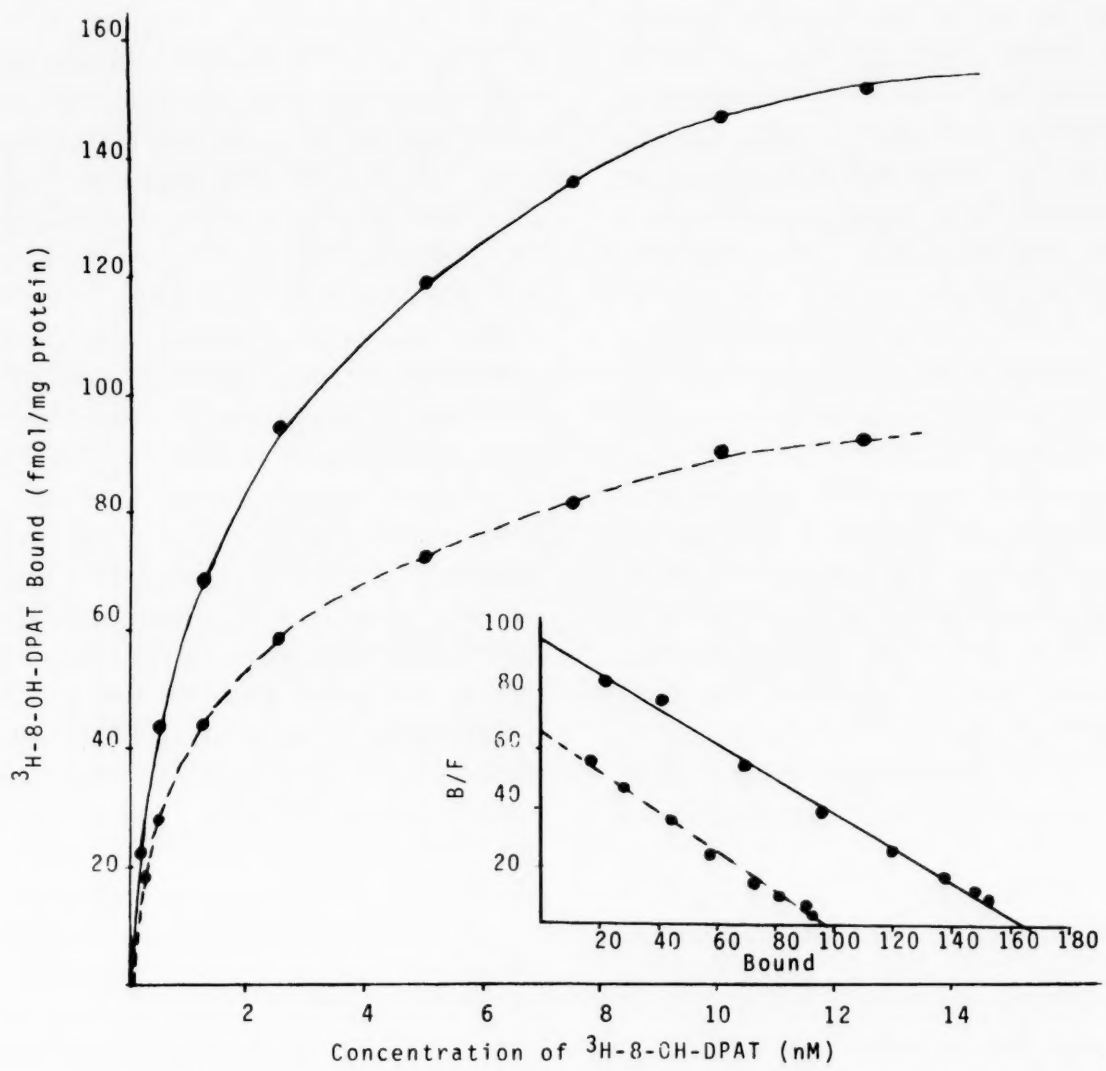

Fig. 1. Saturation isotherm of [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors in rat cerebral cortex (broken line) and hippocampus (solid line). Pooled hippocampi from two rats were used. Each point is the mean of duplicate determinations. Inset, Scatchard plot of the specific binding of [^{3}H]8-OH-DPAT into hippocampal membranes (solid line) and cortical membranes (broken line). Bound = [^{3}H]8-OH-DPAT specifically bound (fmol/mg protein), B/F = Bound over free [^{3}H]8-OH-DPAT (fmol/mg protein × nM). For these particular experiments, binding indices in hippocampus are $K_D = 1.7$ nM, $B_{max} = 164$ fmol/mg protein and C.Co = 0.98 and in cerebral cortex are $K_D = 1.4$ nM, $B_{max} = 95$ fmol/mg protein and C.Co = 0.98.

that chronic treatment with DMI or repeated administration of ECS did not significantly alter the number (B_{max}) or the affinity (K_D) of [^{3}H]8-OH-DPAT binding in rat hippocampus (table 2).

4. Discussion

In order to examine if the mechanism of action of antidepressants is related to their effect on 5-HT_{1A} receptors, we determined the effects of chronic and acute administration with DMI or ECS on 5-HT_{1A} receptor binding sites in rat cortex and hippocampus. We found that chronic DMI or ECS significantly decreased the B_{max} of [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors in cerebral cortex but not in hippocampus. Mizuta and Segawa (1988) reported that chronic treatment with imipramine significantly decreased 5-HT_{1A} receptor binding in rat cortex and hippocampus. However, Newman et al. (1990) reported that chronic treatment with desipramine had no significant effect on 5-HT_{1A} receptor binding in rat hippocampus. Thus, our results are consistent with earlier findings of decreased 5-HT_{1A} receptors in rat cortex after chronic imipramine, as reported by Mizuta and Segawa (1988). Our observation of no change in 5-HT_{1A} receptors in rat hippocampus after chronic DMI is similar to that reported by Newman et al. (1990) but different from Mizuta and Segawa (1988), who observed decreased 5-HT_{1A} receptors in hippocampus after chronic imipramine treatment. In addition, our results also indicate that, similar to DMI, repetitive administration of ECS resulted in decreased 5-HT_{1A} receptor binding in cerebral cortex but not in hippocampus. 5-HT_{1A} receptors have been shown to be located pre- and post synaptically in cortex and postsynaptically in hippocampus. Since we and Newman et al. (1990) did not observe any significant changes in [^{3}H]8-OH-DPAT binding in the hippocampus, this suggests that postsynaptic 5-HT_{1A} receptors are not altered by treatment with DMI or ECS. Thus, one could argue that our data showing a down regulation of 5-HT_{1A} receptors in cortex after chronic antidepressant treatments suggest that this effect is mediated at the presynaptic receptor sites.

Newman and Lerer (1988) reported reduced 5-HT-mediated inhibition of forskolin-stimulated adenylate cyclase in rat hippocampus after chronic DMI or ECS treatment, showing decreased biochemical response of 5-HT_{1A} receptors. These results suggest that 5-HT_{1A} receptors may be down regulated after chronic DMI or ECS treatment, resulting in a decreased biochemical response. However, this is inconsistent with the present results and the findings of Newman et al. (1990), who observed no change in B_{max} of [^{3}H]8-OH-DPAT binding in rat hippocampus. The reason for the discrepancy between the results of response studies of 5-HT_{1A} receptors, i.e. inhibition of forskolin-stimulated adenylate cyclase by 5-HT and [^{3}H]8-OH-DPAT binding, is not clear at this time. Localization of 5-HT_{1A} receptors and inhibitory adenylate cyclase in hippocampus may account for the above discrepancy. Lesiöning studies have indicated a postsynaptic location for 5-HT_{1A} receptors, as measured by [^{3}H]8-OH-DPAT binding in rat hippocampus (Hall et al., 1985). As suggested above, this postsynaptic site may not be affected by antidepressant treatment. Since the majority of 5-HT_{1A} receptors measured by [^{3}H]8-OH-DPAT binding are postsynaptic and if inhibitory adenylate cyclase is located presynaptically (Newman et al., 1990), it is quite possible that changes in the numbers of presynaptic 5-HT_{1A} receptors would not be detected by [^{3}H]8-OH-DPAT binding in the hippocampus. Another possible explanation is that the decrease in forskolin-stimulated adenylate cyclase by 5-HT may be related to changes caused by chronic DMI or ECS treatment at sites distal to the receptors, such as altered receptor coupling to G protein or altered G protein responsiveness, but may not be related to changes in the number or affinity of 5-HT_{1A} receptors.

The fact that similar changes in 5-HT_{1A} receptors were obtained after chronic DMI and ECS administration suggests that the 5-HT_{1A} receptor may be a common site of action for antidepressant treatment as was also found in the case of 5-HT_{1A} receptor response studies (Newman and Lerer, 1988) and behavioral studies (Goodwin et al., 1985, 1987a,b; Desouza et al., 1986). For example presynaptic 5-HT_{1A} receptor-mediated hypothermic response induced by 8-OH-DPAT is attenuated by chronic treatment with both antidepressant drugs and ECS (Green, 1987). Additionally, there is evidence to suggest changes induced by antidepressant treatments is selective for the 5-HT_{1A} receptor as opposed to 5-HT_2 recepter. Ligand binding studies showed that repeated administration of a variety of antidepressant drugs to rats, decreased the number of 5-HT_2 receptors and function in brain (Green, 1987). In contrast to the effects of antidepressant drugs, repeated ECS was found to increase both the number and function of 5-HT_2 receptors in rat and mouse brain (Goodwin et al., 1984; Green, 1987). Thus, both drugs and ECS treatment influence 5-HT_{1A} receptors similarly where as they influence 5-HT_2 receptors differently. This comparison supports the possibility that changes in 5-HT_{1A} receptor may be more asssociated with antidepressant treatment effects than changes in 5-HT_2 subtype receptors.

In summary, our results show that chronic treatment with DMI and ECS results in a down-regulation of 5-HT_{1A} receptors in rat cerebral cortex without influencing these receptors in the hippocampus. Finallly, because both DMI and ECS treatment influence 5-

HT_{1A} receptors in the same direction but 5-HT_2 receptors in opposite directions, we postulate that the 5-HT_{1A} subtype receptor may be common site of action for antidepressant treatments.

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References

DeSouza, R.J., G.M. Goodwin and A.R. Green, 1986, Antidepressant drugs and ECS attenuate the hypothermia and behavioral syndrome following 8-OH-DPAT administration to rats, Br. J. Pharmacol. 89, 523p.

Goodwin, G.M., A.R. Green and P. Johnson, 1984, 5-HT_2 receptor characteristics in frontal cortex and 5-HT_2 receptor mediated head twitch behavior following antidepressant treatment to mice, Br. J. Pharmacol. 83, 235.

Goodwin, G.M., R.J. DeSouza and A.R. Green, 1985, Presynaptic serotonin receptor-mediated response in mice attenuated by antidepressant drugs and electroconvulsive shock, Nature 317, 531.

Goodwin, G.M., R.J. DeSouza and A.R. Green, 1987a, Attenuation by electro convulsive shock and antidepressant drugs of the 5-HT_{1A} receptor mediated hypothermia and serotonin syndrome produced by 8-OH-DPAT in the rat, Psychopharmacology 91, 500.

Goodwin, G.M., R.J. DeSouza, A.R. Green and D.J. Heal, 1987b, The pharmacology of the behavioral and hypothermic responses of rats to 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) Psychopharmacology 91, 506.

Gozlan, H., S.E. Mestikawy, L. Pichat, J. Glowinski and M. Hamon, 1983, Identification of presynaptic serotonin autoreceptors using a new ligand, [^{3}H]-PAT, Nature (London) 305, 140.

Green, A.R., 1987, Evolving concepts on the interactions between antidepressant treatments and monoamine neurotransmitters, Neuropharmacology 26, 815.

Hall, M.D., S.E. Mestikawy, M.B. Emerit, L. Pichat, M. Hamon and H. Gozlan, 1985, [^{3}H]8-hydroxy-2-(di-n-propylamino) tetralin binding to pre- and postsynaptic 5-hydroxytryptamine sites in various regions of the rat brain, J. Neurochem. 44, 1685.

Hamon, M., H. Gozlan, S. Borergoin, S.E. Mestikawy and L. Pichat, 1984, Recent advances in the pharmacology of central serotonergic neurons, Adv. Biol. Psychiat. 14, 12.

Kellar, K.J., C.S. Cascio, J.A. Butler and R.N. Kurtake, 1981, Differential effects of electroconvulsive shock and antidepressant drugs on serotonin-2 receptors in rat brain, Eur. J. Pharmacol. 69, 515.

Kendall, D.A. and S.R. Nahorski, 1985, 5-Hydroxytryptamine-stimulated inositol phospholipid hydrolysis in rat cerebral cortex slices, Pharmacological characterization and effect of antidepressants, J. Pharmacol. Exp. Ther. 233, 473.

Lesch, K.P., J. Disselkamp-Tietze and A. Schmidtke, 1990, 5-HT_{1A} receptor function in depression, Effect of chronic amitriptyline treatment, J. Neural Transm. 80, 157.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

McPherson, G.A., 1985, Analysis of radioligand binding experiments, A collection of computer programs for the IBM PC, J.Pharmacol. Meth. 14, 213.

Meltzer, H.Y. and M.T. Lowy, 1987, The serotonin hypothesis of depression, in: Psychopharmacoloqy: The Third Generation of Progress, ed. H.Y. Meltzer, (Raven Press, New York) p. 513.

Mizuta, T. and T. Segawa, 1988, Chronic effects of imipramine and lithium on postsynaptic 5-HT_{1A} and 5-HT_{1B} sites and on presynaptic 5-HT_3 sites in rat brain, Jap. J. Pharmacol. 47, 107.

Newman, N.E. and B. Lerer, 1988, Chronic electroconvulsive shock and desipramine reduce the degree of inhibition by 5-HT and carbachol of forskolin-stimulated adenylate cyclase in rat hippocampal membranes, Eur. J. Pharmacol. 148, 257.

Newman, N.E., D. Drummer and B. Lerer, 1990, Single and combined effects of desipramine and lithium on serotonergic receptor number and second messenger function in rat brain, J. Pharmacol. Exp. Ther. 252, 826.

Pandey, G.N., S.C. Pandey, P.G. Janicak, R.C. Marks and J.M. Davis, 1990, Platelet serotonin-2 receptor binding sites in depression and suicide, Biol. Psychiat. 28, 215.

Peroutka, S.J. and S.H. Snyder, 1980, Long term antidepressant treatment lowering spiroperidol labelled serotonin receptor binding, Science 210, 88.

Willner, P., 1985, Antidepressants and serotonergic neurotransmission: An integrative review, Psychopharmacology 85, 387.

Wozniak, K.M., C.S. Aulakh, J.L. Hill and D.L. Murphy, 1988, The effect of 8-OH-DPAT on temperature in rat and its modification by chronic antidepressant treatments, Pharmacol. Biochem. Behav. 30, 451.

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Spinal cord substance P mediates the inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area

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A possible role of spinal substance P (SP) in the mediation of signals to inhibit gastric acid secretion by central activation of the sympatho-adrenomedullary system was examined in urethane-anesthetized rats. Intrathecal (i.t.) administration of SP (1–10 nmol) inhibited vagally induced acid output. I.t. administration of spantide, a SP receptor antagonist, reduced the inhibitory effect of 3 nmol SP. I.t. administration of spantide (0.1–1 nmol) blocked the inhibition of vagally induced gastric acid output evoked by electrical stimulation of the preoptic area. Atropine, hexamethonium, phentolamine, propranolol, DL-para-chlorophenylalanine (PCPA) and 5,7-dihydroxytryptamine (5,7-DHT) were without effect. Repeated i.t. administration of 10 nmol SP produced desensitization to the SP-induced inhibitory response on gastric acid output. In these animals, electrical stimulation of the preoptic area did not inhibit vagally induced gastric acid output. These results suggest that electrical stimulation of the preoptic area excites SP-containing neurons in the spinal cord, and a resultant sympatho-adrenomedullary system-mediated inhibition of gastric acid secretion occurs.

Gastric acid secretion; Preoptic area; Substance P; Spinal cord; (Intrathecal injection); (Desensitization)

1. Introduction

It is well known that the splanchnic nerve innervates the gastric sympathetic nerve and the adrenal medulla, and plays an inhibitory role in the regulation of gastric function (Reed et al., 1971; Blair et al., 1975; Yokotani et al., 1983). Centrally applied bombesin (Okuma et al., 1987a), CRF (Taché et al., 1983; Lenz et al., 1985) and prostaglandin E_2 (Yokotani et al., 1988) inhibit gastric acid secretion by activation of the sympatho-adrenomedullary system. Electrical stimulation of the preoptic area, a site of action of bombesin, inhibits the increase in acid output induced by electrical stimulation of the vagus nerve, and this inhibition is mediated by the splanchnic nerve (Okuma et al., 1987b, Okuma and Osumi, 1989). However, the chemical mediators that are released in the spinal cord in response to stimulation of the preoptic area and that regulate splanchnic nerve activity and gastric acid secretion have not been characterized.

Substance P (SP) is contained in the spinal cord. The vasomotor response elicited by injection of kainic acid into the ventral medulla of the rat is blocked by intrathecal (i.t.) administration of a SP antagonist (Loewy and Sawyer 1982). I.t. administration of SP increases plasma levels of catecholamines (Yashpal et al., 1985), and SP or SP agonists increase arterial pressure and heart rate (Keeler et al., 1985; Yashpal et al., 1987).

In the present study, we examined the effect of i.t. administration of SP on vagally induced gastric acid secretion and the effects of spantide and other test substances on the inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area.

2. Materials and methods

Male Wistar rats weighing 250–300 g were maintained in a room at 22–24 °C under a constant day-night rhythm for 7–10 days and given food (laboratory chow, CE-2, Japan CLEA Co.) and tap water ad libitum. Prior to each experiment, all food but not water was withheld for 16 h. Under urethane anesthesia (1.1 g/kg i.p.), the femoral vein was cannulated, and physiological saline was infused at a rate of 1.6 ml/h.

The abdomen was opened by a mid-line incision, and a round-tip cannula (5 cm long, 0.5 cm outer

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diameter) connected to a polyethylene tube was inserted into the stomach via an incision in the duodenum (1 cm distal from the pyloric sphincter). The stomach was flushed with saline, taking care to avoid distention. After repeated washings, 2 ml of solution prewarmed to 38 °C was injected into the stomach at the beginning of each 15-min collection period. This solution was a 1:5 (v/v) mixture of glycine and mannitol adjusted to 300 mOsm and pH 3.5 by addition of 0.1 N HCl, according to Blair et al. (1975). Acid output was determined by titration of gastric samples to pH 7.0 with 0.01 N NaOH, using a pH meter.

To increase gastric acid output, the vagus nerve was electrically stimulated. The vagus nerves were cut bilaterally at the cervical level. The peripheral end of the left vagus nerve was placed on a platinum ring electrode and stimulated continuously throughout the experiments. The stimulus parameters were square-wave pulses of 0.5 ms duration, at 3 Hz, 1 mA, delivered by means of an electric stimulator (Model SEN-7103, Nihon Kohden Ltd., Japan).

The animal was placed in a stereotaxic instrument. Electrical stimulation of the preoptic area was with square-wave pulses of 2 ms duration, at 10 Hz, 0.5 mA, delivered through a bipolar stainless steel electrode (0.15 mm diameter). The coordinates of the preoptic area were AP 6.5, L 0.5, H 2.2, according to the brain atlas of König and Klippel (1963).

After the experiment, the brain was removed, fixed in 10% formalin, and frozen sections were cut (30 μm). These were stained with cresyl violet for microscopic study of the stimulation sites.

Drugs were dissolved in artificial cerebrospinal fluid (CSF). The composition of the CSF, as described by Falcon et al. (1978), was (mg) 7.3 NaCl, 1.9 $NaHCO_3$, 0.3 $MgSO_4$, 0.2 $CaCl_2$, 0.2 NaH_2PO_4 and 0.8 glucose in 1 ml of deionized water. I.t. injections were given through a 15.5-mm long, 27-gauge stainless steel injector needle, which was connected to a 50-μl microsyringe by PE-20 polyethylene tube. The injector needle was inserted at the spinal L5 or T9 level. At the end of an experiment, 5% blue dextran solution was injected i.t. to confirm the localization of the dye within the subarachnoidal space. Drug was delivered in a volume of 10 μl; the catheter was then flushed with 5 μl of CSF over 1 min.

DL-para-Chlorophenylalanine (PCPA) was suspended in saline containing 1% carboxymethyl cellulose sodium salt and administered i.p. at a dose of 400 mg/kg, 3 days before the experiment. 5,7-Dihydroxytryptamine (5,7-DHT) 100 μg and ascorbic acid 50 μg were dissolved in 100 μl CSF. The solution containing 5,7-DHT (100 μl) was continuously infused i.t. (T9) for 2 h under pentobarbital sodium (50 mg/kg i.p.) anesthesia, 10 days before the experiment. Serotonin in the thoracic spinal cord was extracted according to a modified method of Sagara et al. (1988). Tissue samples were weighed and homogenized with 4 ml of 0.4 M perchloric acid and 0.1 ml of 0.1 M EDTA. Nω-Methyl-5-hydroxytryptamine was used as internal standard. After centrifugation at 10000 $\times g$ for 5 min, 2 ml of the supernatant was shaken with 3 ml of chloroform. After centrifugation, the clear supernatants were assayed electrochemically by high-performance liquid chromatography. A pump (Tri-Rotar-II; Japan Spectroscopic Co., Ltd.) and detector (ECD-100, EICOM Co., Ltd.) were used for high-performance liquid chromatography. The analytical conditions were as follows: detector, 0.7 V potential; column, Cosmosil-packed column (ODS) 4.6 $\times$ 150 mm (Nakarai Chemicals Co. Ltd.); mobile phase, 0.1 M phosphate buffer pH 3.1, 50 μM EDTA containing 8% methanol.

Drugs used were: substance P (SP), spantide ([D-Arg^1,D-Trp^{7,9},Leu^{11}]substance P) (Peptide Institute, Inc., Osaka Japan), DL-para-chlorophenylalanine (PCPA), hexamethonium chloride (Nakarai Chemicals), phentolamine mesylate (Ciba-Geigy); atropine sulfate (Merck Chemical Company); 5,7-dihydroxytryptamine creatinine sulfate salt, DL-propranolol hydrochloride, Nω-methyl-5-hydroxytryptamine oxalate (Sigma Chemical Company).

Results are expressed as the means $\pm$ S.E. Student's t-test was used for comparisons between two groups, and Dunnett's test was used for multiple comparisons after a one-way analysis of variance (ANOVA).

3. Results

When the vagus nerve was continuously stimulated (3 Hz, 0.5 ms, 1 mA), gastric acid output increased and reached a steady level within 60 min. I.t. pretreatment of animals with various receptor blocking drugs was done 10 min before electrical stimulation of the preoptic area.

3.1. The effect of i.t. administration of SP on the vagally induced increase in gastric acid output

I.t. administration of SP at the level of L5 even in a dose of 10 nmol did not alter the vagally induced increase in gastric acid output (data not shown). SP was then administered in the upper spinal cord. I.t. administration of SP (1–10 nmol) at the level of T9 dose dependently inhibited the vagally induced increase in gastric acid output (fig. 1). However, i.v. administration of 10 nmol SP did not alter the vagally induced increase in gastric acid output (data not shown).

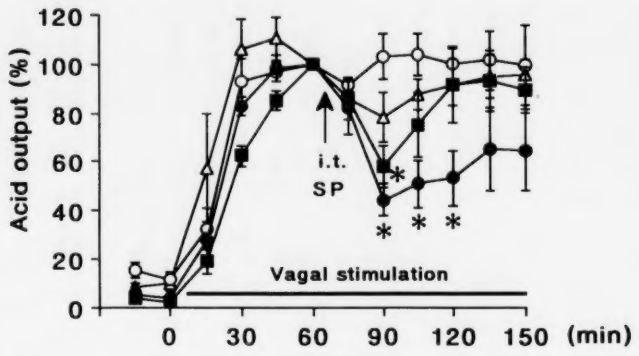

Fig. 1. Effect of i.t. (T9) administration of SP on vagally induced gastric acid output. The left vagus nerve was stimulated at 3 Hz, 0.5 ms, 1 mA. The mean basal gastric acid output was 2.50 ± 0.41 μEq/15 min, and the level 60 min after the start of the vagal stimulation was 34.8 ± 2.7 μEq/15 min (n = 21). (○) Vehicle (n = 6), (△) 1 nmol SP (n = 5), (■) 3 nmol SP (n = 4), (●) 10 nmol SP (n = 6). * $P < 0.05$ (statistically significant difference from the respective control values with vehicle).

3.2. The effect of i.t. pretreatment with spantide, a SP antagonist, on the SP-induced inhibition of gastric acid output

In the animals pretreated with spantide (1 nmol i.t.) (L5), the inhibition of gastric acid output induced by i.t. (T9) administration of 3 nmol SP but not 10 nmol SP was significantly reduced (fig. 2).

3.3. Effects of spantide, adrenoceptor antagonists and acetylcholine receptor antagonists on the inhibition of gastric acid output induced by electrical stimulation of the preoptic area

In control rats given vehicle i.t., electrical stimulation of the preoptic area inhibited the vagally induced gastric acid output by 35% (fig. 3). I.t. (L5) pretreatment with 0.3 nmol spantide significantly reduced the

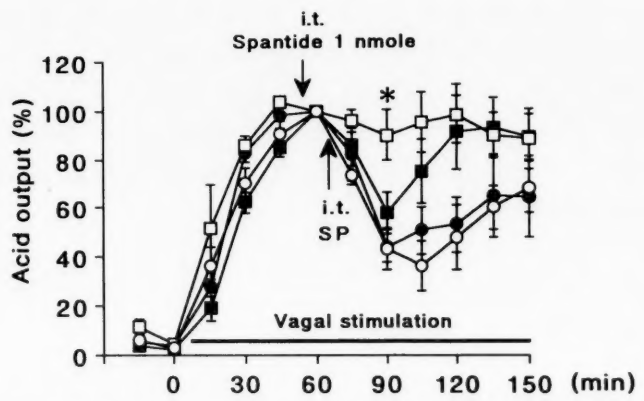

Fig. 2. Effect of i.t. pretreatment with spantide, a SP antagonist, on the inhibition of gastric acid output induced by i.t. administration of SP. The left vagus nerve was stimulated at 3 Hz, 0.5 ms, 1 mA. The mean basal gastric acid output was 1.26 ± 0.23 μEq/15 min, and the level 60 min after the start of the vagal stimulation was 30.0 ± 3.0 μEq/15 min (n = 19). Spantide (1 nmol) was administered i.t. (L5) 10 min before the i.t. (T9) administration of SP. (■) 3 nmol SP (n = 4), (□) 3 nmol SP pretreated with spantide (n = 4), (●) 10 nmol SP (n = 6), (○) 10 nmol SP pretreated with spantide (n = 5). * $P < 0.05$ (statistically significant difference from the values with 3 nmol SP and spantide plus 3 nmol SP).

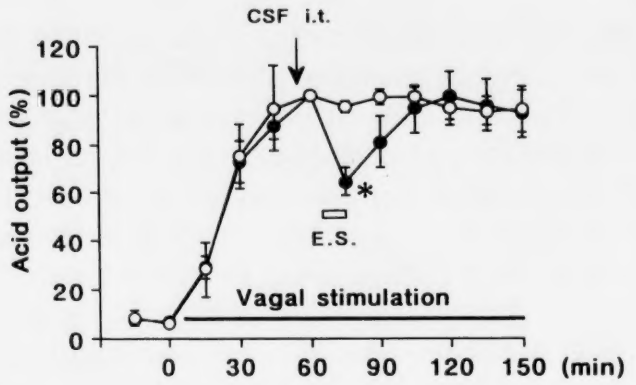

Fig. 3. The effect of electrical stimulation of the preoptic area on vagally induced gastric acid output. The left vagus nerve was stimulated at 3 Hz, 0.5 ms, 1 mA. The mean basal gastric acid output was 2.56 ± 0.31 μEq/15 min, and the level 60 min after the start of the vagal stimulation was 37.5 ± 4.4 μEq/15 min (n = 13). .E.S.: electrical stimulation of the preoptic area (10 Hz, 2 ms, 0.5 mA for 10 min). (○) Control rats (n = 5), (●) rats electrically stimulated in the preoptic area (n = 8). In each group, CSF was administered i.t. (L5) 10 min before the stimulation of the preoptic area. * $P < 0.05$ (statistically significant difference from the respective control values of the non-stimulated group).

inhibition of gastric acid output induced by electrical stimulation of the preoptic area. This electrical stimulation-induced inhibition was abolished by increasing the dose of spantide to 1 nmol (fig. 4). Pretreatment with 1 nmol atropine, 10 nmol hexamethonium, 10 nmol phentolamine or 10 nmol propranolol did not affect the inhibition of gastric acid output induced by stimulation of the preoptic area (table 1).

3.4. Effects of desensitization to SP on the inhibition of gastric acid output induced by i.t. administration of SP and electrical stimulation of the preoptic area

Vagally induced acid output at the 60-min collection period after repeated i.t. (T9) administration of 10

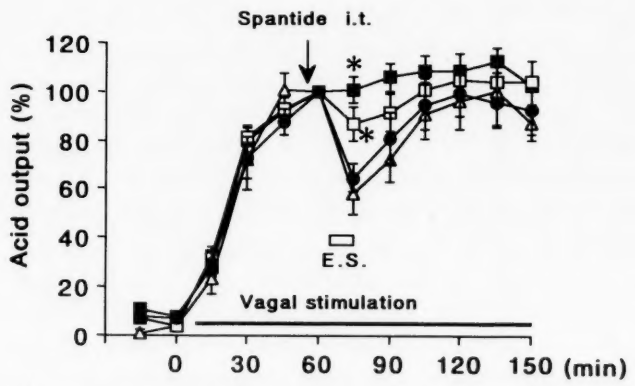

Fig. 4. Effect of i.t. pretreatment with spantide on the inhibition of gastric acid output induced by electrical stimulation of the preoptic area. The left vagus nerve was stimulated at 3 Hz, 0.5 ms, 1 mA. The mean basal gastric acid output was 2.29 ± 0.27 μEq/15 min, and the level 60 min after the start of the vagal stimulation was 33.9 ± 3.0 μEq/15 min (n = 23). E.S.: electrical stimulation of the preoptic area (10 Hz, 2 ms, 0.5 mA for 10 min). Spantide was administered i.t. (L5) 10 min before the stimulation of the preoptic area. (●) Vehicle (n = 8), (△) 0.1 nmol spantide (n = 4), (□) 0.3 nmol spantide (n = 5), (■) 1 nmol spantide (n = 6). * $P < 0.05$ (statistically significant difference from the respective control values obtained with electrical stimulation).

nmol SP was not significantly different from that of respective controls receiving vehicle alone (see legend of fig. 5). Pretreatment with SP (10 nmol × 2) resulted in desensitization to the response of a third administration of SP. Inhibition of vagally induced gastric acid output by 10 nmol SP was not observed in the animals desensitized to SP (fig. 5A). In these animals, the inhibition of gastric acid output induced by electrical stimulation of the preoptic area was abolished (fig. 5B).

3.5. The effect of electrical stimulation of the preoptic area on gastric acid output in PCPA- and 5,7-DHT-pretreated rats

Three days after the administration of PCPA (400 mg/kg i.p.), the serotonin content in the thoracic spinal cord (65.4 ± 8.7 ng/g, n = 5) was reduced to less than

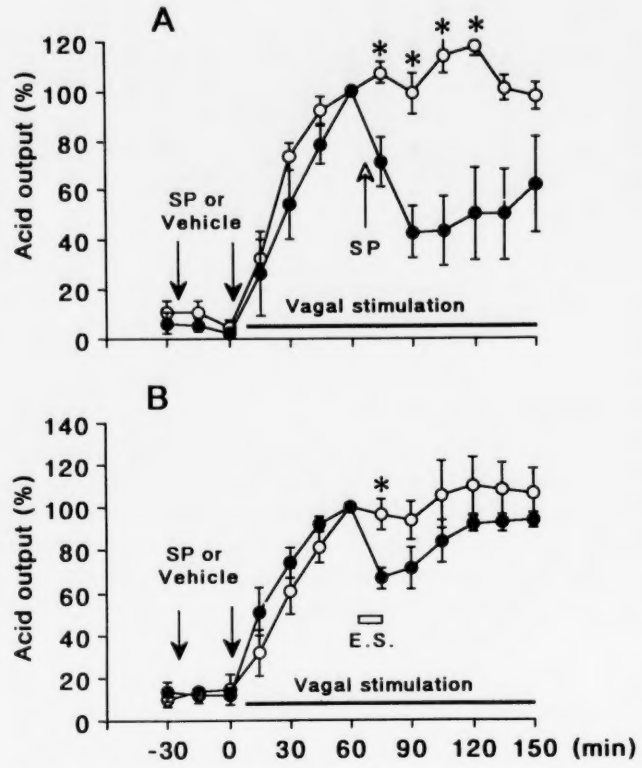

Fig. 5. Effects of desensitization to SP on the inhibition of gastric acid output induced by i.t. (T9) administration of SP (A), and by electrical stimulation of the preoptic area (B). The mean basal gastric acid output was 2.15 ± 0.47 μEq/15 min and the levels at 60 min after repeated i.t. administration of SP 10 nmol and vehicle were 20.5 ± 1.6 μEq/15 min (n = 8) and 24.0 ± 3.3 μEq/15 min (n = 8), respectively. Four rats were used in each experiment. Solid arrows: i.t. administration of vehicle (●) (control group) or 10 nmol SP (○) (SP-pretreated group). Open arrow (in A): i.t. administration of 10 nmol SP in both control and SP-pretreated groups. E.S. (in B): electrical stimulation of the preoptic area (10 Hz, 2 ms, 0.5 mA for 10 min) in both control and SP-pretreated groups. * $P < 0.05$ (statistically significant difference from the respective control values).

TABLE 1

Effects of atropine, hexamethonium, phentolamine, propranolol, PCPA or 5,7-DHT on the inhibition of gastric acid output induced by electrical stimulation of the preoptic area.

Pretreatment	Acid output [a]				n
	Control	n	Electrical stimulation of the preoptic area		
Vehicle	95.1 ± 2.5	5	64.6 ± 6.0 [c]		8
Atropine (1 nmol)	101.5 ± 6.6	3	67.0 ± 11.0 [c]	NS	3
Hexamethonium (10 nmol)	110.4 ± 7.3	4	67.5 ± 4.5 [c]	NS	4
Phentolamine (10 nmol)	112.4 ± 8.6	3	71.6 ± 5.7 [c]	NS	3
Propranolol (10 nmol)	106.0 ± 8.4	4	68.4 ± 4.8 [c]	NS	4
PCPA	109.5 ± 3.0	4	59.3 ± 4.2 [c]	NS	5
5,7-DHT	– [b]		66.3 ± 5.2	NS	5

[a] Acid output obtained in the first 15-min collection period after the treatment (75 min after the start of vagal stimulation) is expressed as a percentage of the respective values just before the treatment (60 min after the start of vagal stimulation). The left vagus nerve was stimulated at 3 Hz, 0.5 ms, 1 mA. Electrical stimulation of the preoptic area was performed at 10 Hz, 2 ms, 0.5 mA for 10 min. Antagonists were administered i.t. (L5) 10 min before stimulation of the preoptic area. PCPA was administered i.p. at a dose of 400 mg/kg, 3 days before the experiment, and 5,7-DHT was administered i.t. (T9) at a dose of 100 μg, 10 days before. [b] Not tested. In the rats pretreated with vehicle injected i.t. (T9) instead of 5,7-DHT, acid output after electrical stimulation was 65.3 ± 6.4% (n = 5), and this value was not significantly different from the value of the group treated with 5,7-DHT. The mean basal gastric acid output and the level 60 min after the start of vagal stimulation in each group were as follows: control, 2.56 ± 0.31, 37.5 ± 4.4 μEq/15 min (n = 13); atropine, 1.19 ± 0.30, 32.7 ± 4.3 μEq/15 min (n = 6); hexamethonium, 1.48 ± 0.51, 28.3 ± 3.3 μEq/15 min (n = 8); phentolamine, 1.93 ± 0.62, 20.7 ± 2.8 μEq/15 min (n = 6); propranolol, 0.55 ± 0.12, 23.9 ± 3.0 μEq/15 min (n = 8); PCPA, 1.76 ± 0.51, 38.4 ± 3.5 μEq/15 min (n = 9), 2.17 ± 0.60, 19.3 ± 5.7 μEq/15 min (n = 5), respectively. [c] $P < 0.05$ (statistically significant difference from the respective control values of the non-stimulated group). NS: no significant difference from values of the group receiving vehicle alone.

15% of that of controls (495 ± 28 ng/g, n = 4). Ten days after the i.t. (T9) administration of 5,7-DHT (100 μg), the serotonin content in the thoracic cord (39.0 ± 10.4 ng/g, n = 5) was reduced to less than 10% of that of controls (495 ± 36 ng/g, n = 5). In these rats depleted of serotonin with PCPA and 5,7-DHT, the inhibition of the vagally induced gastric acid output by electrical stimulation of the preoptic area was not modified (table 1).

4. Discussion

The present study demonstrates that spinal SP-containing neurons are probably involved in the mediation of the central inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area in bilateral cervical vagotomized rats. The spinal regulation of this function is thus similar to the spinal regulation of the sympatho-adrenomedullary system, a notion that is supported by a number of observations. SP-like immunoreactive axons or terminals are found in the intermediolateral cell column (IML), the site of origin

of the sympathetic outflow (Ljungdahl et al., 1978; Ditirro et al., 1981; Holets and Elde, 1982). SP-containing neurons originating from the ventrolateral medulla project to the IML (Helke et al., 1982), and SP binding sites are found in the IML (Maurin et al., 1984; Takano et al., 1985). Sympathetic preganglionic neurons are excited by the iontophoretic application of SP (Gilbey et al., 1983; Backman and Henry, 1984). Sympathetically mediated vasomotor responses elicited from the ventral medulla are blocked by the i.t. administration of SP antagonist (Loewy and Sawyer, 1982). The i.t. administration of SP increases plasma levels of catecholamines (Yashpal et al., 1985), and SP or SP agonists increase arterial pressure and heart rate (Keeler et al., 1985; Yashpal et al., 1987).

Sympathetic preganglionic neurons innervating the stomach originate from T5-T9 in the spinal cord. In the present study, i.t. (T9) administration of SP (1–10 nmol) inhibited the vagally induced gastric acid output. This SP-induced inhibition was antagonized by i.t. (L5) pretreatment with spantide. I.v. administration of SP in the maximum dose used for the i.t. route did not alter the vagally induced gastric acid output. The inhibition of gastric acid output induced by i.t. (T9) SP is therefore probably mediated by activation of SP receptors in the spinal cord at about the T9 level.

The inhibitory effect of SP on vagally induced gastric acid secretion was mimicked by electrical stimulation of the preoptic area. I.t. administration of 1 nmol spantide abolished the inhibition of gastric acid output induced by electrical stimulation of the preoptic area. Administration of atropine, hexamethonium, phentolamine, or propranolol by the same route was without effect.

It is well known that some SP antagonists can produce ischemia of nervous tissues secondary to causing vasoconstriction with subsequent neurotoxic effects (Hökfelt et al., 1981; Post and Paulsson, 1985; Cox et al., 1988; Freedman et al., 1988). However, Helke et al. (1987) reported that at least in the 15- to 20-min time period after i.t. administration of an SP antagonist (3.3 nmol), spinal cord neurons are still functionally responsive to an excitatory input. I.t. administration of spantide at the level of L5 in a dose of 4 nmol but not 1 nmol inhibited the vagally induced increase in gastric acid output, probably due to its non-specific effects (data not shown). Then, 1 nmol of spantide was used. It is unlikely that the response with this relatively low dose of spantide was due to non-specific neurotoxic effects of this agent. SP receptor antagonists can inhibit the action of bombesin by functioning as bombesin receptor antagonists, but they have a 10-fold greater affinity for the SP receptor than the bombesin receptor (Jensen et al., 1988). Bombesin is also present in the spinal cord (Moody et al., 1981; Massary et al., 1983). It has been reported that bombesin injected i.t. inhibits gastric acid output (Yang et al., 1989). Therefore, the possibility still remains that the response found with spantide in the present study is due to the occupation of spinal bombesin receptors by this SP antagonist.

In the next series of experiment, the effects of i.t. pretreatment with a desensitizating regimen of SP on gastric acid secretion were examined. It has been reported that repeated i.t. administration of SP produces desensitization to the hyperalgesic response of SP (Sawynok and Robertson, 1985). In the present study, repeated i.t. administration of 10 nmol SP produced desensitization to the inhibitory effect of SP on gastric acid output. After repeated administration of SP, electrical stimulation of the preoptic area did not inhibit the vagally induced gastric acid output. Therefore, it is likely that the inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area is mediated by spinal SP neurons. Yang et al. (1989) reported that a SP agonist injected i.t. had no effect on gastric acid output. Differences in animal species and methods used may explain the discrepancy between our results and theirs.

Serotonin coexists with SP in medullary neurons (Chan-Palay et al., 1978; Hökfelt et al., 1978; Johansson et al., 1981), and both of these transmitters are present in the IML (Dahlström and Fuxe, 1965; Ljungdahl et al., 1978; Steinbusch 1981). Serotonin increases the firing rate of sympathetic preganglionic neurons (De Groat and Ryall, 1967; Coote et al., 1981; Kadzielawa, 1983; McCall, 1983). In the present study, we used PCPA and 5,7-DHT to deplete the spinal cord of serotonin. PCPA inhibits the biosynthesis of serotonin and 5,7-DHT is a neurotoxin that destroys serotoninergic neuron terminals. However, the inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area was not modified by chemical denervation of spinal serotonergic neurons with PCPA and 5,7-DHT.

Thyrotropin-releasing hormone (TRH)-immunoreactivity and TRH binding sites have been demonstrated in the IML (Lechan et al., 1984; Manaker et al., 1985; Harkness and Brownfield, 1986; Helke et al., 1986). TRH increases the firing rate of sympathetic preganglionic neurons (Backman and Henry, 1984). However, i.t. injection of a TRH agonist does not modify gastric acid output (Ishikawa et al., 1988). We did not examine the role of spinal TRH in the inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area. Based on our present results, however, it is tempting to assume that TRH in the spinal cord does not play a major role in the inhibition of gastric acid secretion induced by electrical stimulation of the preoptic area.

On the basis of observations reported in this study, we conclude that electrical stimulation of the preoptic area excites SP-containing neurons in the spinal cord,

resulting in a sympatho-adrenomedullary system-mediated inhibition of gastric acid secretion.

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References

Backman, S.B. and J.L. Henry, 1984, Effects of substance P and thyrotropin-releasing hormone on sympathetic preganglionic neurones in the upper thoracic intermediolateral nucleus of the cat, Can. J. Physiol. Pharmacol. 62, 248.

Blair, E.L., E.R. Grund, J.D. Reed, D.J. Sanders, G. Sanger and B. Shaw, 1975, The effect of sympathetic nerve stimulation on serum gastrin, gastric acid secretion and mucosal blood flow responses meat extract stimulation in anesthetized cats, J. Physiol. 253, 493.

Chan-Palay, V., G. Jonsson and S.L. Palay, 1978, Serotonin and substance P coexist in neurons of the rat's central nervous system. Proc. Natl. Acad. Sci. U.S.A. 75, 1582.

Coote, J.H., V.H. MacLeod, S. Fleetwood-Walker and M.P. Gilbey, 1981, The response of individual sympathetic preganglionic neurones to microelectrophoretically applied endogenous monoamines, Brain Res. 215, 135.

Cox, B.F., R.L. Schelper, F.M. Faraci and M.J. Brody, 1988, Autonomic, sensory, and motor dysfunction following intrathecal administration of three substance P antagonists, Exp. Brain Res. 70, 61.

Dahlström, A. and K. Fuxe, 1965, Evidence for the existence of monoamine neurons in the central nervous system. II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems, Acta Physiol. Scand. 64 (Suppl. 247), 5.

De Groat, W.C. and R.W. Ryall, 1967, An excitatory action of 5-hydroxytryptamine on sympathetic preganglionic neurones, Exp. Brain Res. 3, 299.

Ditirro, F.J., R.H. Ho and G.F. Martin, 1981, Immunohistochemical localization of substance-P, somatostatin, and methionine-enkephalin in the spinal cord and dorsal root ganglia of the North American opossum, *Didelphis virginiana*, J. Comp. Neurol. 198, 351.

Falcon II, J.C., M.I. Phillips, W.E. Hoffman and M.J. Brody, 1978, Effects of intraventricular angiotensin II mediated by the sympathetic nervous system, Am. J. Physiol. 235, H392.

Freedman, J., C. Post, J. Kåhaström, A. Öhlen, P. Mollenholt, C. Owman, L. Alari and T. Hökfelt, 1988, Vasoconstrictor effects in spinal cord of the substance P antagonist [D-Arg^{1},D-Trp^{7,9},Leu^{11}]-substance P (spantide) and somatostatin and interaction with thyrotropin releasing hormone, Neuroscience 27, 267.

Gilbey, M.P., K.E. McKenna and L.P. Schramm, 1983, Effects of substance P on sympathetic preganglionic neurones, Neurosci. Lett. 41, 157.

Harkness, D.H. and M.S. Brownfield, 1986, Segmental distribution of thyrotropin releasing hormone in rat spinal cord, Brain Res. Bull. 17, 11.

Helke, C.J., J.J. Neil, V.J. Massari and A.D. Loewy, 1982, Substance P neurons project from the ventral medulla to the intermediolateral cell column and ventral horn in the rat, Brain Res. 243, 147.

Helke, C.J., S.C. Sayson, J.R. Keeler and C.G. Charlton, 1986, Thyrotropin-releasing hormone-immunoreactive neurons project from the ventral medulla to the intermediolateral cell column: partial coexistence with serotonin, Brain Res. 381, 1.

Helke, C.J., E.T. Phillips and J.T. O'Neill, 1987, Intrathecal administration of a substance P receptor antagonist: studies on peripheral and central nervous system hemodynamics and on specificity of action, J. Pharmacol. Exp. Ther. 242, 131.

Hökfelt, T., Å, Ljungdahl, H. Steinbusch, A. Verhofstad, G. Nilsson, E. Brodin, B. Pernow and M. Goldstein, 1978, Immunohistochemical evidence of substance P-like immunoreactivity in some 5-hydroxytryptamine-containing neurons in the rat central nervous system. Neuroscience 3, 517.

Hökfelt, T., S. Vincent, L. Hellsten, S. Rosell, K. Folkers, K. Markey, M. Goldstein and C. Cuello, 1981, Immunohistochemical evidence for a 'neurotoxic' action of (D-Pro^{2},D-Trp^{7,9})-substance P, an analogue with substance P antagonistic activity, Acta Physiol. Scand. 113, 571.

Holets, V. and R. Elde, 1982, The differential distribution and relationship of serotoninergic and peptidergic fibers to sympathoadrenal neurons in the intermediolateral cell column of the rat: a combined retrograde axonal transport and immunofluorescence study, Neuroscience 7, 1155.

Ishikawa, T., H. Yang and Y. Taché, 1988, Medullary sites of action of the TRH analogue, RX 77368, for stimulation of gastric acid secretion in the rat, Gastroenterology 95, 1470.

Jensen, R.T., P. Heinz-Erian, S. Mantey, S.W. Jones and J.D. Gardner, 1988, Characterization of ability of various substance P antagonists to inhibit action of bombesin, Am. J. Physiol. 254, G883.

Johansson, O., T. Hökfelt, B. Pernow, S.L. Jeffcoate, N. White, H.W.M. Steinbusch, A.A.J. Verhofstad, P.C. Emson and E. Spindel, 1981, Immunohistochemical support for three putative transmitters in one neuron: coexistence of 5-hydroxytryptamine, substance P- and thyrotropin releasing hormone-like immunoreactivity in medullary neurons projecting to the spinal cord, Neuroscience 6, 1857.

Kadzielawa, K., 1983, Antagonism of the excitatory effects of 5-hydroxytryptamine on sympathetic preganglionic neurons and neurons activated by visceral afferents, Neuropharmacology 22, 19.

Keeler, J.R., C.G. Charlton and C.J. Helke, 1985, Cardiovascular effects of spinal cord substance P: Studies with a stable receptor agonist, J. Pharmacol. Exp. Ther. 233, 755.

König, J.F.R. and R.A. Klippel, 1963, The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain (Williams and Wilkins, Baltimore).

Lechan, R.M., S.B. Snapper, S. Jacobson and I.M.D. Jackson, 1984, The distribution of thyrotropin-releasing hormone (TRH) in the rhesus monkey spinal cord, Peptides 5 (Suppl. 1), 185.

Lenz, H.J., S.E. Hester and M.R. Brown, 1985, Corticotropin-releasing factor. Mechanisms to inhibit gastric acid secretion in conscious dogs, J. Clin. Invest. 75, 889.

Ljungdahl, Å, T. Hökfelt and G. Nilsson, 1978, Distribution of substance P-like immunoreactivity in the central nervous system of the rat I. Cell bodies and nerve terminals, Neuroscience 3, 861.

Loewy, A.D. and W.B. Sawyer, 1982, Substance P antagonist inhibits vasomotor responses elicited from ventral medulla in rat, Brain Res. 245, 379.

Manaker, S., A. Winokur, W.H. Rostène and T.C. Rainbow, 1985, Autoradiographic localization of thyrotropin-releasing hormone receptors in the rat central nervous system, J. Neurosci. 5, 167.

Massary, V.J., Y. Tizabi, C.H. Park, T.W. Moody, C.J. Helke and T.L. O'Donohue, 1983, Distribution and origin of bombesin, substance P and somatostatin in cat spinal cord, Peptides 4, 673.

Maurin, Y., S.H. Buck, J.K. Wamsley, T.F. Burks and H.I. Yamamura, 1984, Light microscopic autoradiographic localization of [^{3}H]substance P binding sites in rat thoracic spinal cord, Life Sci. 34, 1713.

McCall, R.B., 1983, Serotonergic excitation of sympathetic preganglionic neurons: A microiontophoretic study, Brain Res. 289, 121.

Moody, T.W., N.B. Thoa, T.L. O'Donohue and D.M. Jacobowits, 1981, Bombesin-like peptides in rat spinal cord: biochemical characterization, localization and mechanism of release, Life Sci. 29, 2273.

Okuma, Y. and Y. Osumi, 1989, Dual effects on gastric acid secretion of electrical stimulation of anterior parts of the rat hypothalamus, Jap. J. Pharmacol. 49, 37.

Okuma, Y., K. Yokotani and Y. Osumi, 1987a, Sympatho-adrenomedullary system mediation of the bombesin-induced central inhibition of gastric acid secretion, Eur. J. Pharmacol. 139, 73.

Okuma, Y., K. Yokotani and Y Osumi, 1987b, Central site of inhibitory action of bombesin on gastric acid secretion in rats, Jap. J. Pharmacol. 45, 129.

Post, C. and I. Paulsson, 1985, Antinociceptive and neurotoxic actions of substance P analogues in the rat's spinal cord after intrathecal administration, Neurosci. Lett. 57, 159.

Reed, J.D., D.J. Sanders and V. Thorpe, 1971, The effect of splanchnic nerve stimulation on gastric acid secretion and mucosal blood flow in the anaesthetized cat, J. Physiol. 214, 1.

Sagara, Y., Y. Okatani, S. Yamanaka and T. Kiriyama, 1988, Determination of plasma 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan and melatonin by high-performance liquid chromatography with electrochemical detection, J. Chromatogr. 431, 170.

Sawynok, J. and G. Robertson, 1985, Desensitization to substance P following intrathecal injection: A technique for investigating the role of substance P in nociception, Naunyn-Schmiedeb. Arch. Pharmacol. 331, 152.

Steinbusch, H.W.M., 1981, Distribution of serotonin-immunoreactivity in the central nervous system of the rat – cell bodies and terminals, Neuroscience 6, 557.

Taché, Y., Y. Goto, M.W. Gunion, W. Vale, J. River and M. Brown, 1983, Inhibition of gastric acid secretion in rats by intracerebral injection of corticotropin-releasing factor, Science 222, 935.

Takano, Y., W.B. Sawyer and A.D. Loewy, 1985, Substance P mechanisms of the spinal cord related to vasomotor tone in the spontaneously hypertensive rat, Brain Res. 334, 105.

Yang, H., F. Cuttitta, H. Raybould and Y. Taché, 1989, Intrathecal injection of bombesin inhibits gastric acid secretion in the rat, Gastroenterology 96, 1403.

Yashpal, K., S.G. Gauthier and J.L. Henry, 1985, Substance P given intrathecally at the spinal T9 level increases adrenal output of adrenaline and noradrenaline in the rat, Neuroscience 15, 529.

Yashpal, K., S. Gauthier and J.L. Henry, 1987, Substance P given intrathecally at the spinal T9 level increases arterial pressure and heart rate in the rat, J. Autonom. Nerv. Syst. 18, 93.

Yokotani, K., I. Muramatsu, M. Fujiwara and Y. Osumi, 1983, Effects of the sympathoadrenal system on vagally induced gastric acid secretion and mucosal blood flow in rats, J. Pharmacol. Exp. Ther. 224, 436.

Yokotani, K., K. Yokotani, Y. Okuma and Y. Osumi, 1988, Sympathoadrenomedullary system mediation of the prostaglandin E_2-induced central inhibition of gastric acid output in rats, J. Pharmacol. Exp. Ther. 244, 335.

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Delineation of rat kidney α_{2A}- and α_{2B}-adrenoceptors with [^{3}H]RX821002 radioligand binding: computer modelling reveals that guanfacine is an α_{2A}-selective compound

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We developed a method for the simultaneous determination of drug affinity constants for rat α_{2A}- and α_{2B}-adrenoceptor subtypes by using [^{3}H]RX821002 radioligand binding in the kidney. Three competition curves were obtained for each drug: one for the test compound in the absence of ARC 239 (a drug found to have 108-fold higher affinity for α_{2B}- than for α_{2A}-adrenoceptors), one in the presence of ARC 239, and one for ARC 239. It is possible to determine the K_ds of a tested drug for both α_{2A}- and α_{2B}-adrenoceptors by simultaneous computer modelling because of the increased constraint in the calculations given by the inclusion of ARC 239 into the assay. Using this approach, we found guanfacine and oxymetazoline to be highly α_{2A}-selective. The most α_{2B}-selective were ARC 239, prazosin and corynanthine. A number of other drugs, for example UK-14,304, rilmenidine and clonidine, were non-selective or showed minor selectivity for α_{2A}- or α_{2B}-adrenoceptors. Moreover, using Monte Carlo simulations, we showed that the three-curve method gives more accurate estimates of drug binding constants for assays when two receptor sites are present than methods analysing only one competition curve.

α_2-Adrenoceptor subtypes; [^{3}H]RX821002 radioligand binding; Computer modelling; Kidney; (Rat)

1. Introduction

Radioligand binding studies in a variety of mammalian species and tissues have shown that α_2-adrenoceptors exist in at least two pharmacologically distinguishable subtypes, termed α_{2A} and α_{2B} (Cheung et al., 1982; Nahorski et al., 1985; Petrash and Bylund, 1986; Bylund et al., 1988; for review see Bylund, 1988). Moreover, three human (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990) and two rat (Zeng et al., 1990; Chalberg et al., 1990) α_2-adrenoceptors have been cloned, sequenced and expressed in mammalian cell lines. In the course of our studies on α_2-adrenoceptors we recently observed that [^{3}H]RX821002 labelled both α_{2A}- and α_{2B}-adrenoceptors in the rat kidney: 14% of the receptors were of the α_{2A}-subtype and 86% of the α_{2B}-subtype. In the present study we took advantage of this property of [^{3}H]RX821002 to determine simultaneously drug binding constants for α_{2A}- and α_{2B}-adrenoceptors in the kidney, using a computer modelling approach. We evaluated some new α_2-adrenoceptor subtype selective compounds and found that guanfacine was selective for α_{2A}-receptors.

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2. Materials and methods

2.1. Membrane preparation

Male Sprague–Dawley rats weighing 200–250 g were decapitated and their kidneys rapidly excised and homogenized in ice-cold 50 mM Tris-HCl, 5 mM EDTA, 0.1 mM PMSF (phenyl methyl sulphonyl fluoride), 10 μg/ml soybean trypsin inhibitor and 200 μg/ml bacitracin, pH 7.5, using a motor-driven teflon glass homogenizer. The homogenates were spun at 500 $\times g$, and the supernatants were collected and spun at 38 000 $\times g$ for 30 min. The pellets were then washed once with the same buffer and then once again with a similar buffer containing a lower concentration of EDTA (1.5 mM). The final pellets were diluted to protein concentrations of about 2.4 mg protein/ml with 50 mM Tris-HCl, 1.5 mM EDTA, pH 7.5. Aliquots of the membranes were frozen and stored at −80°C until used. Protein was determined according to the method of Lowry et al. (1951).

2.2. Binding studies

Radioligand binding was performed by incubating about 240 μg of the membranes in 150 μl of 1 mM EDTA, 100 μM Gpp(NH)p (guanyl-5′-yl-imido-diphosphate), 140 mM NaCl, 33 mM Tris-HCl, pH 7.5 with [^{3}H]RX821002 and drugs, as indicated, for 1 h at 25°C. Assays were stopped by filtering the incubation mixture through Whatman GF/C fibre glass filterpapers and washing the filters with 20 ml ice-cold 50 mM Tris-Cl, pH 7.5, using a cell harvester (Brandel, Gaithersburg, MD, U.S.A.). The filters were then placed in three millilitres of Ready Protein® scintillation cocktail (Beckman Instruments) and counted in a Beckman LS 1801 β-counter. The counting efficiency was determined by using an external standard and the Beckman Instruments H-number method. Assays were performed in duplicate.

2.3. Statistical methods

Computer modelling of radioligand binding data was performed essentially as described (De Lean et al., 1982; Bergström and Wikberg, 1986) by using programmes coded by us suitable for the Macintosh and IBM-PC computers. In brief the experimental data were fitted to the equation:

$$B_i = \sum_{b=1}^{n} \left(K_{ib} F_i R_b / \left(1 + \sum_{a=1}^{m} K_{ab} F_a \right) \right) + N_i F_i \qquad (1)$$

by using Marquardts algorithm for non-linear regression analysis (Marquardt, 1963). The equation assumes that ligands bound reversibly to independent sites according to the law of mass action. In the formula B_i is the concentration of ligand i bound to the membranes, K_{ib} and K_{ab} the affinity constants for ligand i and a for receptor b, respectively, F_i and F_a the free concentrations of ligands i and a, respectively, R_b the concentration of receptor site b, and N_i the constant ratio of bound/free (i.e. non-specific binding) for ligand i. The free concentrations of ligands were estimated numerically from their total concentrations, as described by De Lean et al. (1982). Residuals were weighted according to the reciprocal of their expected variance using the formula $\text{var}(B) = a_0 + a_1 \cdot B^{a_2}$ (eq. 2) (see De Lean et al., 1982). Constants used were $a_0 = 3 \times 10^{-7}$, $a_1 = 3 \times 10^{-4}$ and $a_2 = 1.5$, which approximated the variance when the reactants were expressed in nanomolar concentrations. In the calculations, all of the curves from a separate experiment were fitted simultaneously by setting the appropriate values for n (number of sites) and m (number of ligands) and arranging the data fed to the computer algorithm appropriately. Moreover, several separate fits were performed for each set of data assuming that one or several sites were present. An F-test comparing the sums of squares for the different models (see De Lean et al., 1982) was then used to judge which model approximated the data the best. This procedure to select the best model is referred to as computer modelling.

2.4. Monte Carlo simulations

For the purpose of testing the all over behaviour of the computer modelling methods, theoretical competiton curves were generated by applying standard methods (Bard, 1974) to eq. (1) as well as by using eq. (2) to calculate the variance for the data points. The same procedure as above was used to estimate the free ligand from the total. For each set of data, three competition curves with 16 assays each were generated in order to simulate data obtained in the present study (see Results for details). The simulated data were then subjected to computer modelling as detailed under Results. Programmes were coded for Turbo Pascal® for PC computers and Light Speed Pascal® for Macintosh.

2.5. Isotopes, drugs and chemicals

[^{3}H]RX821002 (1,4-(6,7(n)-^{3}H)benzodioxan-2-methoxy-2-yl)-2-imidazoline; 51 Ci/mmol) was from Amersham; (−)-adrenaline from Sigma Chemical Co.; ARC 239 from Thomae, Biberach, Germany; BDF 8933 from Beiersdorf AG, Hamburg, Germany; B-HT 933 from Thomae; BRL44408 and BRL41992 from Beecham, Essex, UK; clonidine from Boehringer Ingelheim, Ingelheim/Rhein, Germany; corynanthine from Sigma; FLA 151 (2,6-dichlorobenzylidenamino-3,3-dimethylguanidine HCl), FLA 163 (2-chlorobenzylidenamino-3,3-dimethylguanidine HCl), guanabenz and guanfacine from Astra, Södertälje, Sweden; L-593,029 from Merck, Sharp & Dohme, Rahway, N.J.; (+)- and (−)-medetomidine from Farmos, Turku, Finland; oxymetazoline from Draco, Lund, Sweden; prazosin from Sigma; rauwolscine from Carl Roth, Karlsruhe, Germany; rilmenidine from Servier, Gidy, France; UK-14,304 from Pfizer, Sandwich, England; WB 4101 from Research Biochemicals Inc., Natick, MA; yohimbine from Sigma. All other chemicals were purchased from Merck or Sigma and were of analytical quality.

3. Results

3.1. Determination of dissociation constants of [^{3}H]RX821002 for rat kidney α_{2A}- and α_{2B}-adrenoceptors

Preliminary experiments indicated that [^{3}H]RX 821002 bound to both α_{2A}- and α_{2B}-adrenoceptors in

rat kidney membranes. In order to obtain binding constants for [^{3}H]RX821002 at the two sites, saturation curves for [^{3}H]RX821002 were made by adding various concentrations of the tritiated ligand to the incubation mixtures in the absence and presence of 100 nM ARC 239 or 1 μM BDF 8933 (BDF 8933 was used to define non-specific binding). Moreover, in the same experiment, competition curves for ARC 239 were obtained by adding various concentrations of ARC 239 in the presence of fixed concentrations of [^{3}H]RX821002 ($\sim$ 0.6, 2 and 8 nM, respectively) (fig. 1). All data were then simultaneously subjected to computer modelling. The analysis indicated that a two-site model approximated the data the best, since fitting the data to a two-site model resulted in a drastic reduction in the sums of squares as compared to fitting the data to a one-site model ($P < 0.001$). Moreover, fitting the data to a three-site model did not cause significant further reduction in the sums of squares compared to the two-site model. From nine separate experiments the K_ds of [^{3}H]RX821002 for α_{2A}-sites and α_{2B}-sites were estimated to be 0.678 ± 0.036 and 4.58 ± 0.20 nM (mean $\pm$ S.E.M.; $n = 9$), with capacities 14.6 ± 0.9 and 96.6 ± 5.7 fmol/mg protein, respectively. The K_ds of ARC 239 for α_{2A}-sites and α_{2B}-sites were determined to be 1570 ± 200 and 15.0 ± 1.3 nM ($n = 9$), respectively. These differences in drug affinity are clearly reflected in the curves shown in fig. 1. Firstly, the competition curves shown in fig. 1C are biphasic, reflecting the $\sim$ 100-fold higher affinity of ARC 239 for α_{2B}-adrenoceptors. Secondly, as the concentration of [^{3}H]RX821002 increased from 0.6 to 8 nM, the proportion of the competition curve that correspond to competition at α_{2B}-sites (the upper sigmoid part of the curve) increased relative to that resulting from competition at α_{2A}-sites (the lower sigmoid part of the curve). This is because [^{3}H]RX821002 binds mainly to α_{2A}-adrenoceptors at low concentrations. More α_{2B}-adrenoceptors will become occupied when the concentration of [^{3}H]RX821002 is increased. Moreover, as can be seen from the Scatchard transform of the saturation studies (fig. 1A), the curve representing total specific binding is slightly curved, reflecting the binding of [^{3}H]RX821002 to two sites. When 100 nM ARC 239 was added the transform was seen as a sharply bent curve. This is because 100 nM of ARC 239 will compete substantially with [^{3}H]RX821002 at α_{2B}-sites but not at α_{2A}-sites, thus reducing the slope of the transform at high but not at low concentrations of the radioligand.

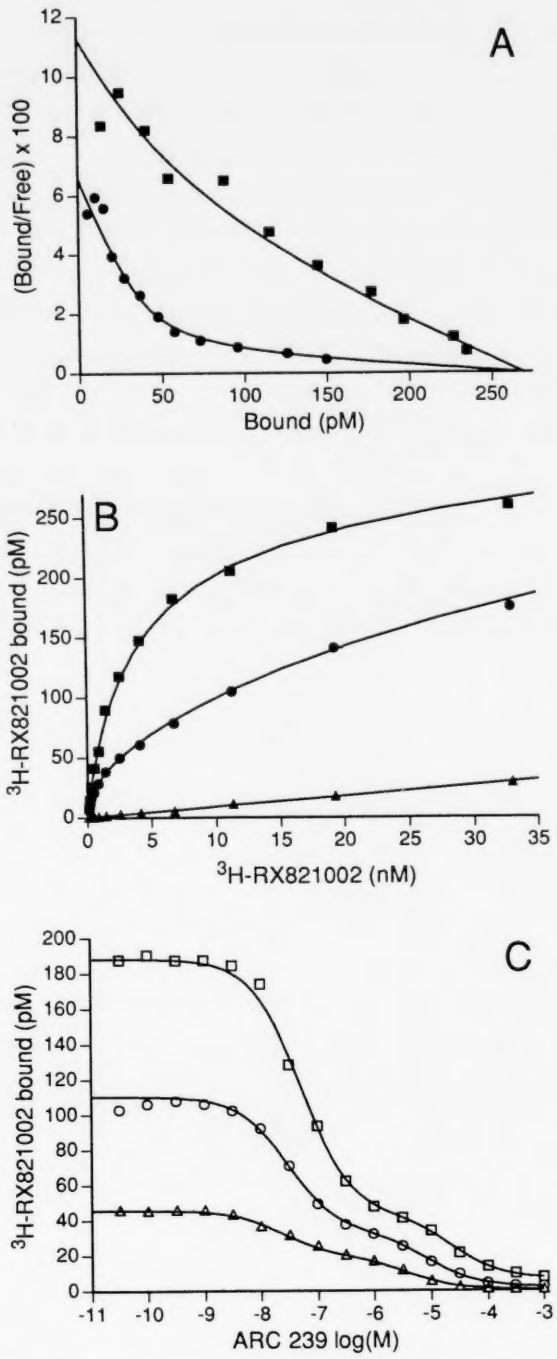

Fig. 1. Saturation and competition experiments, performed on the same day and using the same batch of rat kidney membranes and [^{3}H]RX81002 as labelled ligand. Shown in B are saturation curves for total [^{3}H]RX821002 binding (■), binding of [^{3}H]RX821002 in the presence of 100 nM ARC 239 (●) and in the presence of 1 μM BDF 8933 (▲). Shown in C are competition curves of ARC 239 in the presence of 0.62 (△), 2.4 (○) and 7.6 (□) nM of [^{3}H]RX821002. The lines represent computer-drawn fits from the simultaneous fitting of the data in B and C, assuming that ligands bound reversibly to two independent sites according to the law of mass action. In A is shown the Scatchard transform of the data shown in B; ■ represent the total specific binding and ● the specific binding obtained in the presence of 100 nM ARC 239. The figure represents one experiment out of nine showing essentially the same results.

3.2. Determination of drug dissociation constants by using a three-curve assay

Different drugs were tested in competition studies with a fixed concentration of [^{3}H]RX821002 ($\sim$ 2 nM). Competition curves were constructed for each drug, both in the absence and in the presence of a fixed concentration of ARC 239 (200 nM). A full competition curve for ARC 239 was also obtained (fig. 2). The three curves were then subjected to simultaneous computer modelling.

Figure 2 shows six representative experiments. The competition curves of ARC 239 were strongly biphasic.

Several of the drug competition curves obtained in the absence of ARC 239 were also clearly biphasic or bent. In contrast, all drug competition curves were uniphasic when 200 nM ARC 239 was present. Simultaneous computer modelling of the three curves of each experiment showed that a two-site model fitted the data the best. Thus, fitting the data to a two-site model resulted in highly significant decreases in the sums of squares ($P < 0.001$) compared to when a one-site model was used. Three-site models gave only insignificant reductions in the sums of squares compared to the two-site model.

As can be seen from fig. 2, different patterns were obtained depending on which drug was tested. Figure 2A shows results for prazosin, a drug which is α_{2B}-selective, like ARC 239 (Bylund et al., 1988). In the presence of ARC 239 the biphasicity of the prazosin competition curve was eliminated, so that the curve covered only the low affinity range of the original prazosin competition curve. This pattern indicates the α_{2B}-selectivity of prazosin.

In contrast, the α_{2A}-selective drug oxymetazoline (Bylund et al., 1988) gave a completely different pattern (fig. 2B). The competition curve for oxymetazoline obtained in the presence of ARC 239 covered only the high affinity range of the original oxymetazoline competition curve. This pattern demonstrates the α_{2A}-selectivity of oxymetazoline.

Figure 2C,D show results for (−)-adrenaline and UK-14,304. The competition curves for these compounds obtained in the absence and in the presence of ARC 239, covered exactly the same concentration

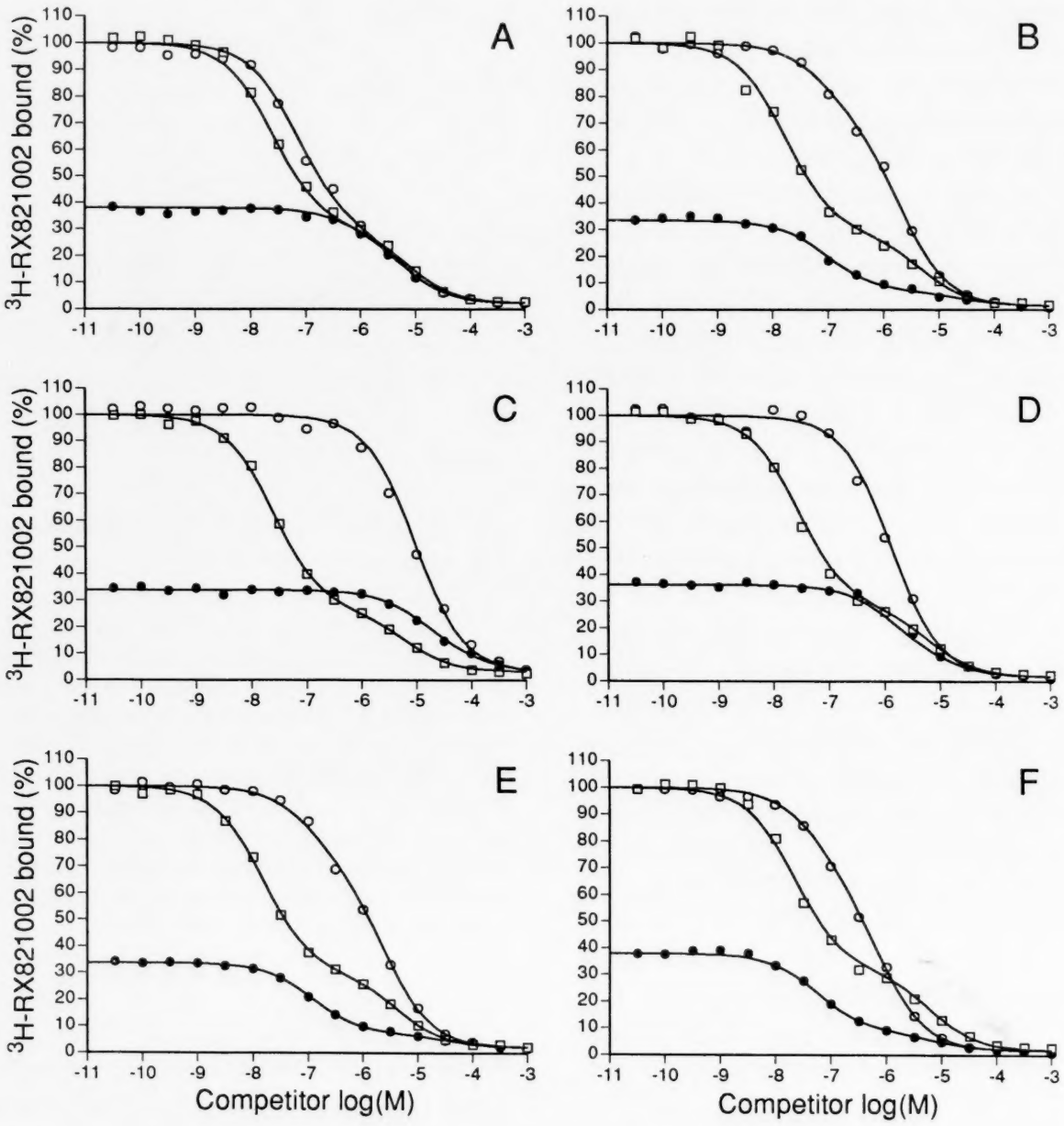

Fig. 2. Competition curves for drugs competing for [^{3}H]RX821002 binding to rat kidney membranes. Membranes were incubated with ~2 nM [^{3}H]RX81002 and various concentrations of ARC 239 (□), various concentrations of a test compound (○) and various concentrations of the test compound in the presence of a fixed concentration (200 nM) of ARC 239 (●). The test compounds were as follows, prazosin (A), oxymetazoline (B), (−)-adrenaline (C), UK-14,304 (D), guanfacine (E) and BRL 44408 (F). The curved lines represent the computer drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to two independent sites according to the law of mass action. The average relative error for a radioligand binding value was estimated to be 4–7% for the different experiments.

ranges. These results thus indicate that (−)-adrenaline and UK-14,304 are non-selective for kidney α_2-adrenoceptor subtypes.

Figure 2E shows results for the α_2-adrenoceptor agonist guanfacine. The pattern obtained was the same as that for oxymetazoline. The pattern for BRL 44408, a drug claimed to be an α_{2A}-selective antagonist (Young et al., 1989), was similar to that for oxymetazoline and guanfacine (fig. 2F).

Table 1 summarizes the results from the analysis of 22 drugs, all assayed in an identical fashion as shown in fig. 2. The most α_{2B}-selective drug was ARC 239, which had a 108-fold selectivity. Prazosin and corynanthine were also α_{2B}-selective (26- and 17-fold, respectively). The most α_{2A}-selective drugs were guanfacine and oxymetazoline (60 and 51-fold, respectively), followed by BRL 44408, which showed 35-fold α_{2A}-selectivity. Rauwolscine showed minor α_{2B}-selectivity (9-fold). Minor α_{2A}-selectivity was found for guanabenz and FLA 151 (20- and 13-fold, respectively). Other drugs showed virtually the same affinity for the α_{2A}- and α_{2B}-adrenoceptors. The data from the computer calculations indicated that the proportions of α_2-adrenoceptor sites labelled by [^{3}H]RX821002 in the kidney membranes were $14 \pm 0.5\%$ for α_{2A} and $86 \pm 0.5\%$ for α_{2B} (n = 33).

3.3. Monte Carlo simulations

In order to verify the ability of the present approach to determine binding constants for drugs in radioligand assays when two sites are present, competition experiments were simulated by using the Monte Carlo approach. In these tests 12 hypothetical compounds with systematic variations in selectivitiy for α_{2A}- and α_{2B}-sites were analysed. Three competition curves were obtained by simulation for each compound, as is detailed under Methods, using the same design as described in paragraph *3.2*. A random normally distributed error was added to the data to simulate experimental variations, as shown in fig. 3. Five simulations were performed for each drug. The data were then analysed by computer modelling, and the results are summarized in table 2. With the three-curve approach to simultaneously analyse the data of each experiment our prediction of drug K_ds by computer modelling was

TABLE 1

Drug dissociation constants (K_ds) obtained from computer modelling of drug competition curves in the rat kidney with ~2 nM [^{3}H]RX821002 as labelled ligand. For each experiment three competition curves were obtained as detailed in the legend to fig. 2 and the data were simultaneously fitted to a model on the assumption that ligands bound to two independent sites according to the law of mass action (e.g. the three-curve approach). All tests indicated that a two site model gave statistically significant ($P < 0.001$) better fits than a one-site model. A shows data for the drugs found to be α_{2A}-selective, and B shows data for drugs found to be α_{2B}-selective. The K_d ratios are estimated from the ratios calculated from the separate experiments. Numbers are given as means ± S.E.M. of 2–33 separate experiments each performed with duplicate determinations. n = number of experiments.

Drug	n	K_d (nM)		Ratio (α_{2B}/α_{2A})
		α_{2A}	α_{2B}	
(A)				
UK-14,304	4	410 ± 63	636 ± 49	1.7 ± 0.3
(+)-Medetomidine	2	18.3 ± 2.3	30.8 ± 4.6	1.7 ± 0.5
(−)-Adrenaline	3	3110 ± 671	4690 ± 868	1.8 ± 0.8
B-HT 933	2	8820 ± 424	16700 ± 2600	1.9 ± 0.4
(−)-Medetomidine	2	44.2 ± 3.2	116 ± 1	2.6 ± 0.2
BDF 8933	3	0.536 ± 0.050	1.38 ± 0.19	2.6 ± 0.6
FLA 151	2	189 ± 16	2400 ± 70	13 ± 2
Guanabenz	2	24.8 ± 6.8	463 ± 15	20 ± 5
BRL 44408	2	13.6 ± 0.4	480 ± 25	35 ± 1
Oxymetazoline	4	35.4 ± 5.8	1660 ± 112	51 ± 9
Guanfacine	4	30.9 ± 3.7	1850 ± 179	60 ± 3
(B)				Ratio (α_{2A}/α_{2B})
ARC 239	33	1530 ± 90	14.4 ± 0.7	108 ± 5
Prazosin	3	1360 ± 78	52.1 ± 3.0	26 ± 3
Corynanthine	2	8570 ± 400	509 ± 49	17 ± 1
Rauwolscine	4	67.6 ± 13.1	12.6 ± 5.2	8.7 ± 2.9
Yohimbine	3	57.7 ± 2.4	15.7 ± 1.4	3.7 ± 0.2
WB 4101	3	184 ± 3	52.8 ± 2.4	3.5 ± 0.2
BRL 41992	2	78.0 ± 3.6	34.4 ± 4.9	2.3 ± 0.4
FLA 163	2	1160 ± 30	557 ± 90	2.1 ± 0.3
L-657,743	2	2.51 ± 0.24	1.34 ± 0.05	1.9 ± 0.2
Rilmenidine	3	8290 ± 2850	5240 ± 1750	1.6 ± 0.1
Clonidine	2	84.4 ± 4.5	71.3 ± 1.4	1.2 ± 0.1

TABLE 2

Results from computer modelling of simulated competition curves of the type shown in fig. 3. Each simulation included one hypothetical drug for which affinities were selected as shown under *Actual* K_d. Three competition curves were obtained for each simulation, one for the hypothetical drug, one simulating ARC 239 and one for the hypothetical drug in the presence of 200 nM of ARC 239. Simulations were performed using receptor concentrations and drug affinities close to those found experimentally (see the table and the legend to fig. 3 for constants). For each hypothetical drug simulations were repeated five times. The data of each separate simulation were then subjected to computer modelling. When all three curves in each set of data were analysed, the K_ds of all hypothetical drugs could be predicted as shown under *Three-curve* K_d. When only data for the competition curve of the hypothetical drugs were used for computer modelling, drug K_ds were predicted only for drugs with high selectivity ratios, as shown under *One-curve* K_d. The reason that the one-curve approch failed for low ratio drugs was: (1) The computer modelling programme signalled 'Ill-conditioning' due to a singular matrix rendering computations mathematically not feasible, (2) the K_ds recovered were erratic with grossly (i.e. more than 10-fold) differing results, or (3) a two site model did not yield a significantly better fit than a one site model. n.c. = not calculable. A = α_{2A}-site. B = α_{2B}-site. Number within parentheses denotes the relative standard error given in %. It should be observed that the average error of the simulated data was about double that of the real experiments (Cf. legends to figs. 2 and 3.)

Ratio	Site	K_d (nM)		
		Actual	Three-curve	One-curve
1/32	A	800	873 (8.6)	1410 (22)
	B	25	25 (8.3)	21 (24)
1/16	A	600	598 (7.4)	548 (31)
	B	38	33 (7.9)	25 (17)
1/8	A	400	387 (5.8)	636 (22)
	B	50	45 (10)	45 (16)
1/4	A	300	326 (11)	n.c.
	B	75	72 (4.8)	n.c.
1/2	A	200	200 (11)	n.c.
	B	100	104 (4.8)	n.c.
1	A	150	148 (35)	n.c.
	B	150	135 (7.2)	n.c.
2	A	100	113 (6.6)	n.c.
	B	200	188 (2.2)	n.c.
4	A	75	73 (5.0)	n.c.
	B	300	317 (7.1)	n.c.
8	A	50	54 (7.8)	n.c.
	B	400	388 (5.3)	n.c.
16	A	38	41 (3.8)	n.c.
	B	600	590 (2.7)	n.c.
32	A	25	24 (6.6)	40 (35)
	B	800	810 (2.3)	750 (20)
64	A	19	17 (5.2)	11 (31)
	B	1200	1190 (1.5)	1160 (12)

good. For comparison, the data were also analysed by computer modelling of the competition curve of the tested drug (e.g. a standard one-curve experimental design). As can be seen from table 2, this procedure predicted binding constants only for compounds with high selectivity ratios. Moreover, for these compounds the errors in the estimates of drug K_ds were much higher than when the three-curve approach was used.

4. Discussion

We have presented a method for the simultaneous determination of drug K_ds for rat α_{2A}- and α_{2B}-adrenoceptors. Our data confirm that [^{3}H]RX821002 is a good radioligand for α_2-adrenoceptors (Langin et al., 1989; Galitzky et al., 1990). The ligand shows negligible non-specific binding (less than 3% of the total binding) thereby providing a high signal to noise ratio. Our results show that [^{3}H]RX821002 labels kidney α_{2A}-adrenoceptors with ~7-fold higher affinity than it labels α_{2B}-adrenoceptors. In comparison to [^{3}H]yohimbine and [^{3}H]rauwolscine, which are two popular radioligands used to assay α_2-adrenoceptors, [^{3}H]RX 821002 shows superior properties because of its higher affinity and much lower non-specific binding. Moreover, our data indicate that rauwolscine and yohimbine may be slightly α_{2B}-selective for rat kidney α_2-adreno-

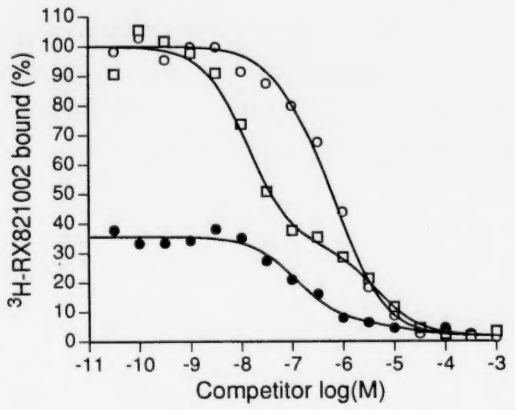

Fig. 3. Example of a computer simulated three-curve competition assay. Shown are competition curves for a hypothetical drug (○), a simulated ARC 239 competition curve (□) and a competition curve for the hypothetical drug in the presence of 200 nM of ARC 239 (●). Constants used for the calculations were, for eq. 1 R1 (α_{2A}) = 2.7×10^{-2} nM, R2 (α_{2B}) = 1.7×10^{-1} nM, K11 ([^{3}H]RX82102-α_{2A}) = 1.47 nM^{-1}, K12 ([^{3}H]RX82102-α_{2B}) = 0.218 nM^{-1}, K21 (ARC 239-α_{2A}) = 6.53×10^{-4} nM^{-1}, K22 (ARC 239-α_{2B}) = 6.94×10^{-2} nM^{-1}, K31 (hypothetical drug-α_{2A}) = 4×10^{-2} nM^{-1}, K32 (hypothetical drug-α_{2B}) = 1.25×10^{-3} nM^{-1}. The error introduced in the simulated curves generated an average relative error of 13%.

ceptors (table 1). This makes it difficult to use [^{3}H]yohimbine or [^{3}H]rauwolscine to assay both α_2-adrenoceptor subtypes in the kidney as binding to α_{2B}-adrenoceptors will predominate.

With respect to nomenclature we chose to refer to the α_2-adrenoceptors of the kidney as 'rat α_{2A}' and 'rat α_{2B}'. The classification is according to the original classification of Bylund (1985), i.e. α_{2A}-types have low affinity for prazosin and α_{2B}-types high affinity for prazosin, whereas the opposite is valid for oxymetazoline. However, there is reason to believe that there are species differences for α_2-adrenoceptors (see for example Michel et al., 1989b; Brown et al., 1990; Connaughton and Docherty, 1990) and that this has added confusion to the nomenclature of α_2-adrenoceptor subtypes. We therefore prefer to refer to our receptors as 'rat-types'. Besides, we regard our nomenclature as provisional since, in the future, molecular cloning data and pharmacological data will serve as a basis for a rational nomenclature of α_2-adrenoceptor subtypes.

The increased accuracy of the three-curve method now described is accomplished by the inclusion into each experiment of ARC 239, a drug which we found to have 108-fold higher affinity for rat α_{2B}- than for α_{2A}-adrenoceptors. The inclusion of a full competition curve for ARC 239 accurately defines the proportions of α_{2A}- and α_{2B}-adrenoceptor sites when the data is subjected to computer modelling. This will in turn help to define the K_ds for the tested drug since the magnitude of the proportion of sites will not be dependent solely on data from the competition curve for the tested drug. Moreover, the affinity of the tested drug for α_{2A}-adrenoceptors is unambigously derived from the competition curve for the drug obtained in the presence of 200 nM ARC 239. At this concentration ARC 239 will nearly completely block the binding of [^{3}H]RX81002 to α_{2B}-adrenoceptors, without affecting binding to α_{2A}-adrenoceptors. Since all K_d values are determined by simultaneous computer modelling of all curves, the K_d value of a tested drug for α_{2B}-adrenoceptors can be determined accurately, because the possible values for this parameter are restricted when the other parameters are defined accurately.

Our method appears to be useful to determine drug K_ds of both agonists and antagonists for the α_2-adrenoceptor subtypes. It is well known that agonists may bind to a high affinity conformation of the α_2-adrenoceptors consisting of a ternary complex with agonist, receptor and a G-protein, as well as to a low affinity conformation, consisting of the receptor uncoupled from its G-protein (see Hoffman et al., 1980a,b; Hoffman and Lefkowitz, 1982; Van Megen et al., 1990). In the present study we obtained competition curves using an incubation buffer containing EDTA, Gpp(NH)p and NaCl. The reason for this was that a number of previous studies have indicated that these conditions will eliminate the high affinity conformation of the α_2-adrenoceptor, so that agonists only bind to the low affinity conformation of the receptor (Snavely and Insel, 1982; U'Prichard, 1984; Nunnari et al., 1987; Schloos et al., 1987; Michel et al., 1989a). Snavely and Insel (1982) showed that these conditions rendered (−)epinephrine competition curves in the rat kidney resolvable into only one-site fits when they used [^{3}H]yohimbine as radioligand in conjunction with computer modelling procedures similar to those described in the present paper. ([^{3}H]yohimbine is expected to label predominantely α_{2B}-adrenoceptors in the rat kidney because of its slightly higher affinity for this receptor type and the predominance of α_{2B}-adrenoceptors in the organ). Moreover, we have recently shown for a pure rat α_{2A}-adrenoceptor system (the rat spinal cord) that EDTA, Gpp(NH)p and NaCl render (−)epinephrine competition curves resolvable into one-site fits (Uhlén and Wikberg, submitted manuscript). The data of our present study are consistent with the notion that high affinity conformations of α_2-adrenoceptors for agonists do not interfere in the assays because the two-site model described the experimental data equally well for both agonists and antagonists (cf. the computer fitted curves for the agonists (−)-adrenaline, UK-14,304, oxymetazoline and guanfacine with the computer fitted curves for the antagonists prazosin and BRL 44408 in fig. 2).

We have shown that guanfacine is selective for α_{2A}-adrenoceptors in the rat kidney. Guanfacine has never before been reported to be an α_2-adrenoceptor subtype selective drug. Another substance which has been described as α_{2A}-selective is oxymetazoline (Bylund, 1988). The α_{2A}-selectivity of guanfacine was similar to

that of oxymetazoline. Apart from these two compounds, there do not appear to be many α_{2A}-selective compounds. Young et al. (1989) found that BRL 44408 was 72-fold more selective for α_{2A}-adrenoceptors than for α_{2B}-adrenoceptors in the rat cerebral cortex, using [^{3}H]rauwolscine as labelled ligand. These investigators found oxymetazoline to be 31-fold more selective for α_{2A}-adrenoceptors than for α_{2B}-adrenoceptors when assayed in the same way. In our present study we found BRL 44408 to be 35-fold selective for the α_{2A}- compared to the α_{2B}-adrenoceptors in the rat kidney, whereas oxymetazoline was 51-fold more selective. Thus, our data is consistent with that of Young et al. (1989). Nevertheless, since gene cloning data show that there are more than two species of α_2-adrenoceptors (Lomasney et al., 1990; Zeng et al., 1990; Chalberg et al., 1990), more studies are required to compare various α_2-adrenoceptors before one can establish their similar or dissimilar natures with certainty.

Previous studies of the pharmacology of guanfacine do not give much indication that its α_{2A}-selectivity plays a role in its pharmacological properties (see Van Zwieten, 1986; Scholtysik and Fetkovska, 1987). However, in a recent study we observed that whereas the antinociceptive effect of intrathecal UK-14,304 was highly susceptible to up-regulation after chronic depletion of spinal cord noradrenaline, the antinociceptive effect of intrathecal guanfacine was not (Uhlén et al., 1990). Since our data (table 1) indicate that UK-14,304 is non-selective, it is tempting to speculate that the difference in susceptibility for the regulation of guanfacine and UK-14,304 effects is caused by the difference in their selectivity for α_2-adrenoceptor subtypes.

It was interesting that whereas FLA 151 was clearly α_{2A}-adrenoceptor selective, FLA 163 was non-selective (table 1). Both compounds are structurally similar guanidinium analogues, the only difference being that FLA 151 possess two chlorine atoms at position 2 and 6 whereas FLA 163 contains only one at the 2 position of the aromatic ring. Moreover, guanfacine and guanabenz, which were found to be α_{2A}-adrenoceptor selective (table 1), are structurally similar to FLA 151 as they also have 2,6-chlorine substitutions in their aromatic rings. This information should be useful for the future design of new α_2-adrenoceptor subtype-selective ligands.

In summary, we have developed a method for the simultaneous determination of drug binding constants for α_{2A}- and α_{2B}-adrenoceptors, using a computer modelling approach. Our experimental design takes advantage of the labelling of both α_{2A}- and α_{2B}-adrenoceptors in the rat kidney by [^{3}H]RX821002 and of the ability of ARC 239 to markedly distinguish between these sites. By simultaneous computer analysis of drug and ARC 239 competition curves, as well as drug competition curves obtained in the presence of a fixed concentration of ARC 239, the K_ds of tested drugs can be determined accurately. Using this approach we evaluated some new α_2-adrenoceptor subtype selective drugs. Of these, guanfacine was found to be strongly α_{2A}-selective.

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References

Bard, Y., 1974, Nonlinear Parameter Estimation (Academic Press, New York) p. 210.

Bergström, A. and J.E.S. Wikberg, 1986, Guanine nucleotides regulates both agonist and antagonist binding to cod brain α_1-adrenoceptors, Acta Pharmacol. Toxicol. 59, 270.

Brown, C.M., A.C. MacKinnon, J.C. McGrath, M. Spedding and A.T. Kilpatrick, 1990, Heterogeniety of α_2-adrenoceptors in rat cortex but not human platelets can be defined by 8-OH-DPAT, RU 24969 and methysergide, Br. J. Pharmacol. 99, 481.

Bylund, D.B., 1985, Heterogeneity of alpha-2 adrenergic receptors, Pharmacol. Biochem. Behav. 22, 835.

Bylund, D.B., 1988, Subtypes of α_2-adrenoceptors: Pharmacological and molcular biological evidence converge, Trends Pharmacol. Sci. 9, 355.

Bylund, D.B., C. Ray-Prenger and T.J. Murphy, 1988, Alpha-2A and alpha-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype, J. Pharmacol. Exp. Ther. 245, 600.

Chalberg, S.C., T. Duda, J.A. Rhine and R.K. Sharma, 1990, Molecular cloning, sequencing and expression of an α_2-adrenergic receptor complementary DNA from rat brain, Mol. Cell. Biochem. 97, 161.

Cheung, Y.-D., D.B. Barnett and S.R. Nahorski, 1982, [^{3}H]Rauwolscine and [^{3}H]yohimbine binding to rat cerebral and human platelet membranes; Possible heterogeneity of α_2-adrenoceptors, Eur. J. Pharmacol. 84, 79.

Connaughton, S. and J.R. Docherty, 1990, Functional evidence for heterogeneity of peripheral prejunctional α_2-adrenoceptors, Br. J. Pharmacol. 101, 285.

De Lean, A., A.M. Hancock and R.I. Lefkowitz, 1982, Validation and statistical analysis of radioligand binding data for mixtures of pharmacological receptor subtypes, Mol. Pharmacol. 21, 5.

Galitzky, J., L. Larrouy, M. Berlan and M. Lafontan, 1990, New tools for human fat cell alpha-2A adrenoceptor characterization. Identification on membranes and on intact cells using the new antagonist [^{3}H]RX821002, J. Pharmacol. Exp. Ther. 252, 312.

Hoffman, B.B. and R.I. Lefkowitz, 1982, Agonist interactions with α-adrenergic receptors, J. Cardiovasc. Pharmacol. 4, S14.

Hoffman, B.B., T. Michel, D. Mullikin-Kilpatrick, R.J. Lefkowitz, M.E.M. Tolbert, H. Gilman and I.N. Fain, 1980a, Agonist versus antagonist binding to alpha-adrenergic receptors, Proc. Natl. Acad. Sci. U.S.A. 77, 4569.

Hoffman, B.B., D. Mullikin-Kilpatrick and R.J. Lefkowitz, 1980b, Heterogeneity of radioligand binding to alpha-adrenergic receptors. Analysis of guanine nucleotide regulation of agonist binding in relation to receptor subtypes, J. Biol. Chem. 255, 4645.

Kobilka, B.K., H. Matsui, T.S. Kobilka, T.L. Yang-Feng, U. Francke, M.G. Caron, R.J. Lefkowitz and J.W. Regan, 1987, Cloning, sequencing, and expression of the gene coding for the human platelet alpha$_2$-adrenergic receptor, Science 238, 650.

Langin, D., M. Lafontan, M.R. Stillings and H. Paris, 1989, [^{3}H]RX821002 a new tool for the identification of α_{2A}-adrenoceptors, Eur. J. Pharmacol. 167, 95.

Lomasney, J.W., W. Lorenz, L.F. Allen, K. King, J.W. Regan, T.L. Yang-Feng, M.G. Caron and R.J. Lefkowitz, 1990, Expansion of the α_2-adrenergic receptor family: Cloning and characterization of a human α_2-adrenergic receptor subtype, the gene for which is located on chromosome 2, Proc. Natl. Acad. Sci. U.S.A. 87, 5094.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193, 265.

Marquardt, D.W., 1963, An algorithm for least squares estimation on non-linear parameters, J. Soc. Ind. Appl. Math. 2, 431.

Michel, M.C., O.-E. Brodde, B. Schnepel, J. Behrendt, R. Tschada, H.J. Motulsy and P.A. Insel, 1989a, [^{3}H]Idazoxan and some other α_2-adrenergic drugs also bind with high affinity to a non-adrenergic site, Mol. Pharmacol. 35, 324.

Michel, A.D., D.N. Loury and R.L. Whiting, 1989b, Differences between the α_2-adrenoceptor in rat submaxillary gland and the α_{2A}- and α_{2B}-adrenoceptor subtypes, Br. J. Pharmacol. 98, 890.

Nahorski, S.R., D.B. Barnett and Y.-D. Cheung, 1985, α-Adrenoceptor-effector coupling, affinity states or heterogeniety of the α_2-adrenoceptor?, Clin. Sci. 68 (Suppl. 10), 39S.

Nunnari, J.M., M.G. Repaske, S. Brandon, E.J. Cragoe, Jr. and L.E. Limbird, 1987, Regulation of porcine brain α_2-adrenergic receptors by Na^+,H^+ and inhibitors of Na^+/H^+ exchange, J. Biol. Chem. 262, 12387.

Petrash, A.C. and D.B. Bylund, 1986, Alpha-2 adrenergic receptor subtypes indicated by ^{3}H-yohimbine binding in human brain, Life Sci. 38, 2129.

Regan, J.W., T.S. Kobilka, T.L. Yang-Feng, M.G. Caron, R.J. Lefkowitz and B.K. Kobilka, 1988, Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype, Proc. Natl. Acad. Sci. U.S.A. 85, 6301.

Schloos, J., A.S. Wellstein and D. Palm, 1987, Agonist binding at α_2-adrenoceptors of human platelets using ^{3}H-UK-14,304: regulation by Gpp(NH)p and cations, Naunyn-Schmiedeb. Arch. Pharmacol. 336, 48.

Scholtysik, G. and N. Fetkovska, 1987, Pharmacology of guanfacine, Cor Vasa 29 (4-Suppl. 1), S11.

Snavely, M.D. and P.A. Insel, 1982, Characterization of α-adrenergic receptor subtypes in the rat renal cortex. Differential regulation of α_1- and α_2-adrenergic receptors by guanyl nucleotides and Na^+, Mol. Pharmacol. 22, 532.

Uhlén, S., M.-L. Persson, L. Alari, C. Post, K.L. Axelsson and J.E.S. Wikberg, 1990, Antinociceptive actions of α_2-adrenoceptor agonists in the rat spinal cord: evidence for antinociceptive α_2-adrenoceptor subtypes and dissociation of antinociceptive α_2-adrenoceptors from cyclic AMP, J. Neurochem. 55, 1905.

U'Prichard, DC, 1984, Biochemical characteristics and regulation of brain α_2-adrenoceptors, Ann. N.Y. Acad. Sci. 430, 55.

Van Megen, Y.J.B., J.F. Rodrigues de Miranda, A.B.M. Klaassen, B.T.R. Wentges and C.A.M. Van Ginneken, 1990, α_2-Adrenoceptors in homogenates of rat nasal mucosa, Eur. J. Pharmacol. 182, 561.

Van Zwieten, P.A., 1986, Overview of α_2-adrenoceptor agonists with a central action, Am. J. Cardiol. 57, 3.

Young, P., J. Berge, H. Chapman and M.A. Cawthorne, 1989, Novel α_2-adrenoceptor antagonists show selectivity for α_{2A}- and α_{2B}-adrenoceptor subtypes, Eur. J. Pharmacol. 168, 381.

Zeng, D., J.K. Harrison, D.D. D'Angelo, C.M. Barber, A.L. Tucker, Z. Lu and K.R. Lynch, 1990, Molecular characterization of a rat α_{2B}-adrenergic receptor, Proc. Natl. Acad. Sci. U.S.A. 87, 3102.

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Concentration-related effects of extracellular application of ATP on the action potential and membrane currents of the guinea-pig vas deferens

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The ionic mechanisms underlying concentration-related alterations in the action potential configuration caused by ATP were studied using preparations of the guinea-pig vas deferens voltage-clamped by a double sucrose gap method. Under current-clamp conditions, ATP at concentration of 1.6 μM enhanced the rates of rise and of repolarisation of the action potential whereas at concentration of 1.6 mM it reduced both rates. Under voltage-clamp conditions, lower concentrations increased the maximum inward Ca current without altering kinetics or reversal potential. Higher concentrations reduced the maximum inward Ca current with slowing of rates of activations and inactivation, but also caused a negative shift in reversal potential without affecting conductance. These results suggest that a low ATP concentration activates the voltage-dependent Ca current channels and that the action of a high ATP concentration is related to the internal Ca ion concentration.

Vas deferens (guinea-pig); ATP; Ca^{2+} currents (voltage-dependent); Ca^{2+} mobilization (intracellular)

1. Introduction

There is growing understanding of the role of regulatory mechanisms of adenosine triphosphate (ATP) in the voltage-dependent ionic current system in various smooth muscles (urinary bladder: Klöckner and Isenberg, 1985; intestine: Ohya et al., 1987; artery: Ohya and Sperelakis, 1989). Most studies using smooth muscle cells used the effects of intracellular application of ATP on the voltage-dependent Ca currents to clarify the dependence of Ca channel activities on cytoplasmic nucleotide metabolism as was described for the typical Ca excitable cells such as cardiac muscles (Irisawa and Kokubun, 1983) and neurones (Doroshenko et al., 1984).

While ATP-mediated (purinergic) neurotransmission (see review by Burnstock, 1981) and the role of ATP in energy metabolism have been well studied, little is known about the regulation of voltage-dependent ionic channels by ATP applied to purinergic innervated smooth muscle cells such as the vas deferens (Sneddon and Westfall, 1984). This lack is especially true for ATP applied to the outside of the membrane as demonstrated for cardiac muscles (Goto et al., 1977; Flitney and Singh, 1980), skeletal muscles (Hume and Thomas, 1988) and neurones (Yatani et al., 1982).

It is generally accepted that an ionic mechanism underlies the membrane depolarization induced by ATP via P_2-purinoceptor activation (guinea-pig: Wakui and Inomata, 1985; Ji and Inomata, 1990; rat: Nakazawa and Matsuki, 1978; Friel, 1988; DDT_1 MF-2 cells: Molleman et al., 1989). It is, however not certain that, in the vas deferens muscles, extracellular application of ATP can affect the voltage-dependent Ca channel current responsible for generation of the action potential possibly mediated through P_2-purinoceptor activation.

Therefore, we now investigated the effects of extracellular application of ATP on the action potential and membrane currents of the guinea-pig vas deferens muscle cells by means of a double sucrose gap method. Brief accounts of this work have been published (Inomata and Wakui, 1984).

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2. Materials and methods

Male guinea-pig weighing about 200–300 g were used. Small bundles of longitudinal smooth muscle

tissue were isolated from the vas deferens. Typical preparations were 0.4 mm in diameter and 5–8 mm in length.

Details of the double sucrose gap voltage clamp method have been given earlier (Inomata and Kao, 1976, 1985; Kao and Inomata, 1986; Yamagata et al., 1983). Membrane current and voltage were displayed on a storage oscilloscope (Tektronix 5111) for photographing and were monitored throughout the experiment on a pen-writing recorder (Sanei Rectigraph 8S). Some membrane properties are expressed on the basis of unit capacitance to facilitate comparison.

The reference Krebs medium had following ionic composition (mM): Na^+ 137.3; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; HCO_3^- 15.5; $H_2PO_4^-$ 1.2; Cl^- 133.9; glucose 11.5. A tetraethylammonium chloride loading medium (TEA medium) was prepared by replacement of 68 mM NaCl with an equimolar amount of TEA. The solution was gassed with 95% O_2 and 5% CO_2, and its pH was adjusted to 7.4. The perfusate maintained at 32°C flowed continously through the test node from a valve-selectable reservoir containing the control or test medium. Drugs to be tested were added to the medium reservoir to give the desired final concentrations.

The drugs used were adenosine triphosphate disodium (ATP) (Kowa, Japan) as purinoceptor agonist, phentolamine (Ciba-Geigy) or prazosin (Pfizer) as α-adrenoceptor blocker, dl-propranolol (ICI) as β-adrenoceptor blocker, and indomethacin (Sigma) as prostaglandin synthetase inhibitor. The significance of the results was evaluated using a paired Student's t-test and was set at $P < 0.05$.

3. Results

We showed earlier that ATP applied exogenously at a physiological concentration to the vas deferens muscle in normal Krebs medium usually causes depolarization of the membrane with a transient spike burst (Wakui and Inomata, 1985). However, although ATP at concentration lower than 5 μM produced no change in the resting membrane potential, it caused a prominent change in the action potential elicited by electrical stimulation. Figure 1 (upper panels) shows the changes of the action potential configuration before, during and after exposure to 1.6 μM ATP of a preparation in normal Krebs medium. A significant increase of the spike amplitude and shortening of the spike duration were generally observed (fig. 1A and B). These effects were stable for at least 20 min during prolonged exposure, but a further increase of the spike amplitude was seen immediately after washout of the agonist (fig. 1C), followed by a prompt return to the control value. Following these observations the preparation was re-exposed to 1.6 μM ATP under voltage-clamp conditions. Figure 1 (lower panels) shows the membrane currents recorded before and during exposure to the agonist. In agreement with above characteristic changes in the action potential due to ATP, not only the peak inward Ca current, but also the peak outward K current (peak I_K) and the concomitant tail current were appreciably increased during the action of agonist (fig. 1F compared with fig. 1E). However, there was no detectable change in the late outward K current (late I_K) and in the base holding current.

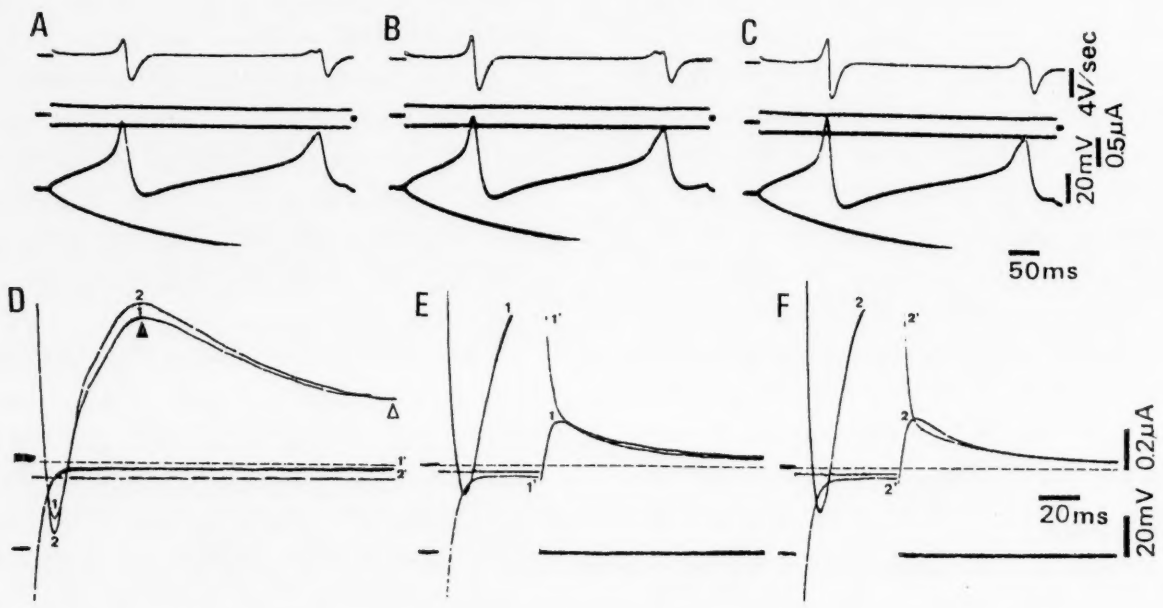

Fig. 1. Membrane responses of vas deferens to constant-current and constant-voltage steps, before (control) and during (test) exposure to 1.6 μM ATP in normal Krebs medium. A to F, records made from same preparation. A, action potential evoked in control; B, in test medium; C, within 30 s after washout. D, membrane currents elicited by 450-ms polarizing step pulses from holding potential of −45 mV in control (traces 1 and 1′) and in test medium (traces 2 and 2′). Filled triangles denotes peak I_K; open triangle, late I_K. E and F, membrane currents elicited by 50-ms polarizing step pulses followed by tail and residue capacitative currents after repolarization in control and in test medium, respectively. Note an increased tail current after repolarization with an increased inward Ca current in test medium.

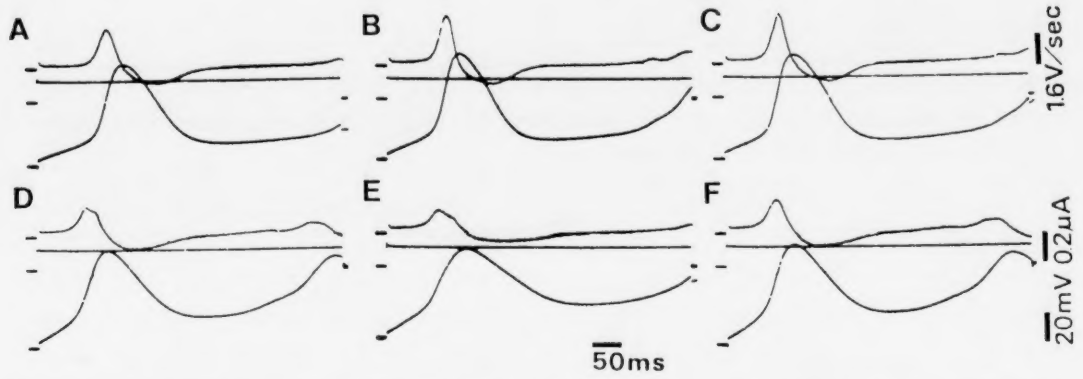

Fig. 2. Membrane potential responses to two concentrations of ATP in TEA medium. A, B and C, action potentials evoked before, during and after exposure to 1.6 μM ATP; D, E and F, before, during and after exposure to 1.6 mM ATP, respectively. Note the enhanced action potential within 1 min after washout followed by a depressed action potential at a higher ATP concentration as shown in F.

3.1. Concentration-dependent effects of ATP on the action potential in TEA medium

The change in membrane currents responsible for generation of the action potential due to ATP in normal Krebs medium introduced above was then analyzed further in TEA medium.

3.1.1. Resting and action potentials

When half of the external Na ion concentration was replaced by TEA ions in normal Krebs medium in an attempt to suppress the I_K, a slight depolarization with repetitive spike discharge appeared. In nodal tissue where repetitive activity was not present, action potential with a plateau on the repolarization phase were elicited by depolarizing current pulses. Figure 2, regarding a preparation in TEA medium, shows the main effects of two different concentrations of ATP on the resting and active membranes. Application of ATP at the low concentration of 1.6 μM caused no change in the resting membrane potential, but significantly enhanced the amplitude of the action potential as well as the rates of rise and of repolarization. Exposure for 2 min produced steady state effects (fig. 2B). These effects remained unaltered during continous exposure (fig. 2C). After observation of the action potential for 10 min after washout of the agonist, the preparation was re-exposed to ATP at the high concentration of 1.6 mM. This concentration produced a rapid depolarization of the membrane, which gradually declined on continuous exposure to the agonist (data not shown). When the membrane had returned to the same level as the control resting potential, the electrically elicited action potential was marked by a prolonged duration and reduced amplitude (fig. 2E). Washout of the agonist was followed by an enhanced action potential (fig. 2F). These similar changes in action potential due to ATP at two concentrations were observed in nine experiments each one with a different preparation. The data are summerized in tables 1 and 2.

3.2. Concentration dependence of the response of the ionic currents to ATP in TEA medium

The nature of the ionic basis of the above concentration-dependent changes in the action potential configuration due to ATP in TEA medium can be better appreciated when the ionic currents are analyzed under voltage-clamp conditions.

TABLE 1

Effects of 1.6 μM ATP on electrical properties of vas deferens in TEA^+ medium. All values are means ± S.E.M. with number of preparation in parentheses. Item 3 is time from peak of action potential to 50% decay. Item 5 is reversal potential of Ca^{2+} current (I_{Ca}). Item 6 negative sign indicates inward current. Item 7 is maximum chord conductance of Ca^{2+} current defined as $I_{Ca}/(E-E_a)$. Item 8 is time-to-peak of I_{Ca}. Item 9 is time constant of inactivation. Item 10 is maximum chord conductance of I_K defined as $I_K/(E-E_K)$. All values are means ± S.E.M., with number of samples in parentheses.

	68 mM TEA	68 mM TEA + ATP	P
(1) Resting potential (mV)	−47.2 ± 1.9 (10)	−47.9 ± 1.6 (10)	0.35
(2) Spike amplitude (mV)	61.3 ± 2.5 (10)	63.2 ± 1.9 (10)	0.1
(3) Spike duration (ms)	107.4 ± 19.4 (9)	102.9 ± 19.6 (9)	< 0.05
(4) Rise phase dV/dt (V/s)	1.71 ± 0.14 (10)	1.94 ± 0.15 (10)	< 0.01
(5) E_a (mV)	+19.9 ± 2.8 (9)	+21.3 ± 3.1 (9)	0.25
(6) Maximum I_a (μA/μF)	−0.72 ± 0.07 (9)	−0.88 ± 0.07 (9)	0.001
(7) $\bar{g}_a$ (μS/μF)	44.5 ± 7.3 (8)	50.4 ± 7.3 (8)	< 0.05
(8) t_a (ms)	39.6 ± 6.2 (8)	40.1 ± 5.0 (8)	0.3
(9) τ_h (ms)	53.4 ± 5.6 (5)	53.1 ± 5.9 (5)	0.35
(10) $\bar{g}_K$ (μS/μF)	17.0 ± 1.3 (8)	20.2 ± 1.5 (8)	0.025

TABLE 2

Effects of 1.6 mM ATP on electrical properties of vas deferens in TEA^+ medium. Same conventions as in table 1. P value for Student's t-test with paired comparison. The reversal potential of I_K, E_K was estimated by tail current analysis (as described earlier by Inomata and Kao (1985)). All values are means ± S.E.M., with number of samples in parentheses.

	68 mM TEA	68 mM TEA + ATP	P
(1) Resting potential (mV)	−49.2 ± 1.2 (15)	−44.5 ± 1.8 (15)	< 0.01
(2) Spike amplitude (mV)	61.6 ± 2.6 (11)	55.7 ± 1.3 (11)	< 0.01
(3) Spike duration (ms)	99.6 ± 14.0 (9)	119.0 ± 16.6 (9)	< 0.01
(4) Rise phase dV/dt (V/s)	2.04 ± 0.17 (10)	1.49 ± 0.20 (10)	< 0.01
(5) E_a (mV)	+22.2 ± 2.8 (9)	+17.7 ± 3.4 (9)	0.03
(6) Maximum I_a (μA/μF)	−0.75 ± 0.16 (8)	0.61 ± 0.12 (8)	0.03
(7) $\bar{g}_a$ (μS/μF)	34.9 ± 4.9 (6)	33.6 ± 4.1 (6)	0.3
(8) t_a (ms)	48.7 ± 4.6 (5)	58.2 ± 4.4 (6)	0.01
(9) τ_h (ms)	50.2 ± 2.8 (5)	58.0 ± 4.2 (5)	0.01
(10) $\bar{g}_K$ (μS/μF)	9.9 ± 1.7 (6)	10.5 ± 1.9 (6)	0.1

3.2.1. Early and late currents

Figure 3 shows typical current recorded from each preparation before and during the application of ATP at two different concentrations and the associated current-voltage relations. After the stable recording of a family of membrane currents at various levels under control conditions, this family of currents was usually recorded periodically under the test conditions. Under control conditions, the membrane currents associated with a smaller depolarization were characterized by an early inward Ca current (I_{Ca}) which was rapidly activated and decayed slowly (fig. 3A,D). A slowly developing outward K current (I_K) appeared at the membrane potential at which the amplitude of the I_{Ca} was maximal. With further depolarization, the I_{Ca} decreased with a marked enhancement of the I_K (fig. 3B,E). Application of 1.6 μM ATP significantly increased the I_{Ca} and the I_K at all potentials tested (fig. 3A,B). These effects always occurred without any detectable change in the base holding current and persisted during continous application of the agonist. These results are consistent with similar findings in normal Krebs

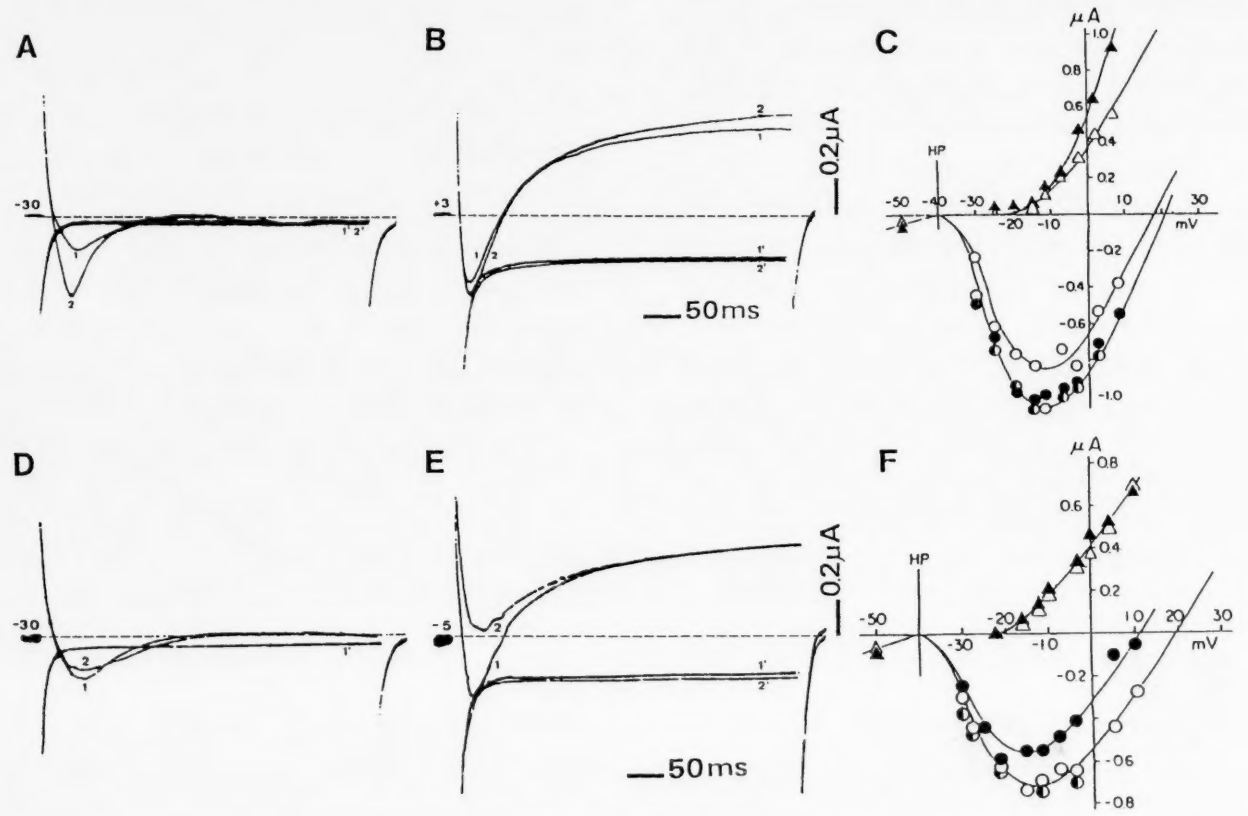

Fig. 3. Membrane current responses to two concentrations of ATP in TEA medium and current-voltage relations. A and B, membrane currents obtained before and during exposure to 1.6 μM ATP, respectively; D and E, before and during exposure to 1.6 mM ATP, respectively. Current records in upper and in lower panels obtained from different preparations. Current traces 1 and 1′, in control; traces 2 and 2′, in test medium. Depolarizing (1 and 2) and hyperpolarizing responses (1′ and 2′) to identical steps, in control or in test medium were double-exposed on the same negatives for ease of comparison. Zero current indicated by broken line. Number on the left of each frame denotes membrane potential attained during depolarizing step from a holding potential of −40 mV. In C and F, current-voltage relations established from current records in the upper and in lower panels, respectively. Open symbols, before; filled symbols, during exposure to ATP. Half-filled symbols, 2–5 min after washout. Note leakage conductance increased by ATP, as evident from superimposed hyperpolarizing current responses (1′ and 2′); the increase was greater with a higher concentration.

medium as described above. On the other hand, application of 1.6 mM ATP caused multiple changes in the membrane currents, depending on exposure time. In the initial response, within 4 min, the base holding current shifted inwardly then returned to the original level, while the I_{Ca} was reduced at all potentials tested. This reduced effect became more pronounced with time, reaching steady state within 6–10 min, but the I_K that appeared at any of the potentials tested remained unchanged throughout exposure to agonist (fig. 3D,E). These features were more marked in the current-voltage relations for each ionic current under control and test conditions (fig. 3C,F), that is, with a low concentration of ATP the maximum I_{Ca} was larger, and the reversal potential of the I_{Ca} and the potential where the I_{Ca} reached its maximum amplitude remained almost fixed, while, with a high concentration, the maximum I_{Ca} was smaller and both potentials were shifted towards negative. The concentration-dependent changes of membrane currents described above were similar in an extended series of sixteen preparations (tables 1 and 2, items 5 and 6).

In different series of experiments (seven preparations) the effects of ATP were re-examined in the presence of α- and β-adrenoceptor and muscarinic antagonists. Neither 5 μM phentolamine, 5 μM propranolol, nor 1 μM atropine had any significant effect on the action of ATP, ruling out the possiblity that this action could be mediated by the release of endogenous neurotransmitter and/or by direct combination with their receptor (data not shown).

3.2.2. Conductances of the Ca and K current channels

When the membrane conductances for the Ca and K current channels are plotted on a logarithmic scale as function of the membrane potential, the effects of ATP on membrane conductance are clearer. Figure 4 illustrates the voltage-conductance relations obtained from two series of experiments on the effects of two different concentrations of ATP. The presence of 1.6 μM ATP raised the curves for Ca (g_a) and K conductances (g_K) without any significant shift in the voltage axis. That is, in agreement with the augumented g_a at any level of membrane potential, the g_K also was increased (fig. 4A). The calculated values of the maximum conductances for both ionic current channels were thereby increased by about 13 and 12%, respectively (table 1, items 7 and 10). On the other hand, the presence of 1.6 mM ATP causes no detectable changes in the voltage-conductance curve for the Ca current channels, indicating that the maximum Ca conductance ($\bar{g}_a$) remained about the same. There was, however, an appreciable increase in the g_K for the membrane potential between -15 and $+5$ mV, but the increases were less prominent for membrane potentials more positive than $+10$ mV, resulting in no significant change in maximum conductance ($\bar{g}_K$) (fig. 4B; table 2, items 7 and 10).

3.2.3. Activation and inactivation

In order to evaluate the kinetic aspects of the I_{Ca} under different conditions, we attempted to compare the rates of activation and inactivation obtained from the same group of preparations under control and test conditions. The time to peak of the I_{Ca} was used as measure of the rate of activation. As is evident from the current records of figs. 3 and 5, the peak of the I_{Ca} at all voltages tested during exposure to 1.6 μM ATP was reached at about the same time as under control conditions, whereas the peak of the I_{Ca} during expo-

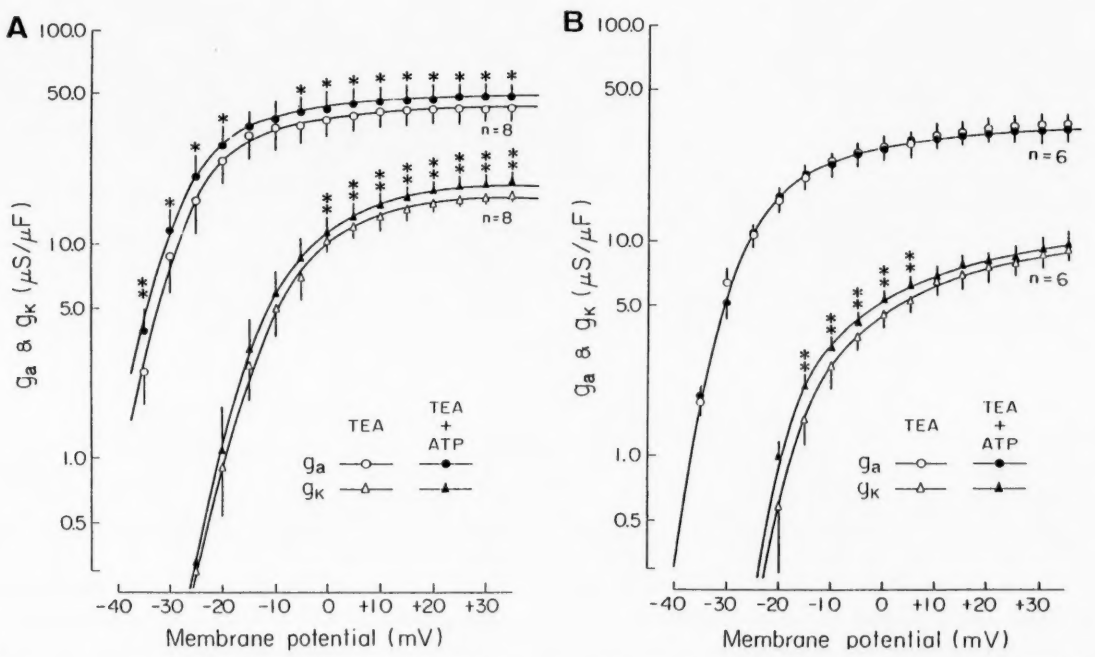

Fig. 4. Conductances of Ca and K current channels as a function of membrane potential before (open symbols) and during (filled symbols) exposure to two concentrations of ATP. A, average g_a (circles) and g_K (triangles) of eight preparations before and during action of 1.6 μM ATP; B, average g_a and g_K of six preparations before and during the action of 1.6 mM ATP. Mean $\pm$ S.E.M. of eight or six preparations are shown. * $P < 0.05$; ** $P < 0.03$ (compared to corresponding controls).

sure to 1.6 mM ATP was reached more slowly than under control conditions.

When a relatively small depolarization step was maintained, the I_{Ca} decayed after reaching a maximum. The rate of decay was estimated as time constant of inactivation from its semilogarithmic plot (fig. 5). The time course of decay is usually composed of two exponential terms: a fast and a slow current component as shown in fig. 5 (A). This decay is progressively faster with increasing depolarization, but higher depolarizations tend to cause an unfavourable outward current, which masks the slow component of the I_{Ca}. Thus, for convenience, regarding the voltage relation of the time constant on inactivation of the I_{Ca}, only the fast component was estimated, except in the case of the slow component as depicted in legend for fig. 5. There was a clear difference in decay on application of 1.6 mM ATP as contrasted to that on application of 1.6 μM ATP. In the preparation exposed to 1.6 μM ATP, the magnitude of the fast component which was obtained by extrapolating the fitted exponential to $t = 0$ was increased with the same constant as in the control, whereas in the preparation exposed to 1.6 mM ATP, the magnitude of the fast component was reduced with a slower time constant. The average values of the time constants at 0 mV obtained from five preparations exposed to 1.6 μM ATP remained unaltered (table 2, item 9), while the constants obtained from five other preparations exposed to 1.6 mM increased significantly (table 2, item 9).

4. Discussion

The results of the present study indicate that, in the guinea-pig vas deferens muscle, extracellularly applied ATP has a strikingly different, concentration-dependent effect on voltage-dependent ionic current during generation of the action potential; that is, at a concentration too low to induce membrane depolarization, ATP causes marked enhancement in voltage-dependent Ca (I_{Ca}) and K current (I_K), whereas at a concentration sufficiently high to induce membrane depolarization I_{Ca} is reduced though there is a slight enhancement of I_K.

The response of these parameters to different ATP concentrations explained the contrasting concentration-dependent effects of agonists on action potential configuration. At a low concentration (1.6 μM), ATP causes an enhancement in the maximal rate of rise and the amplitude of the action potential (table 1, items 2 and 4) with the result that the maximum inward I_{Ca} as well as the Ca channel conductance (g_a) are significantly greater (table 1, items 6 and 7). Combined with enhanced I_{Ca}, the enhancement in the late I_K caused by increasing conductance (table 1, item 10) can account for the increase in the rate of repolarization of the action potential (table 1, item 3). At a high concentration (1.6 mM), ATP causes a reduction in the maximal rate of rise and in the amplitude of the action potential (table 2, items 2 and 4) by affecting the kinetics of activation and inactivation of I_{Ca} (table 2,

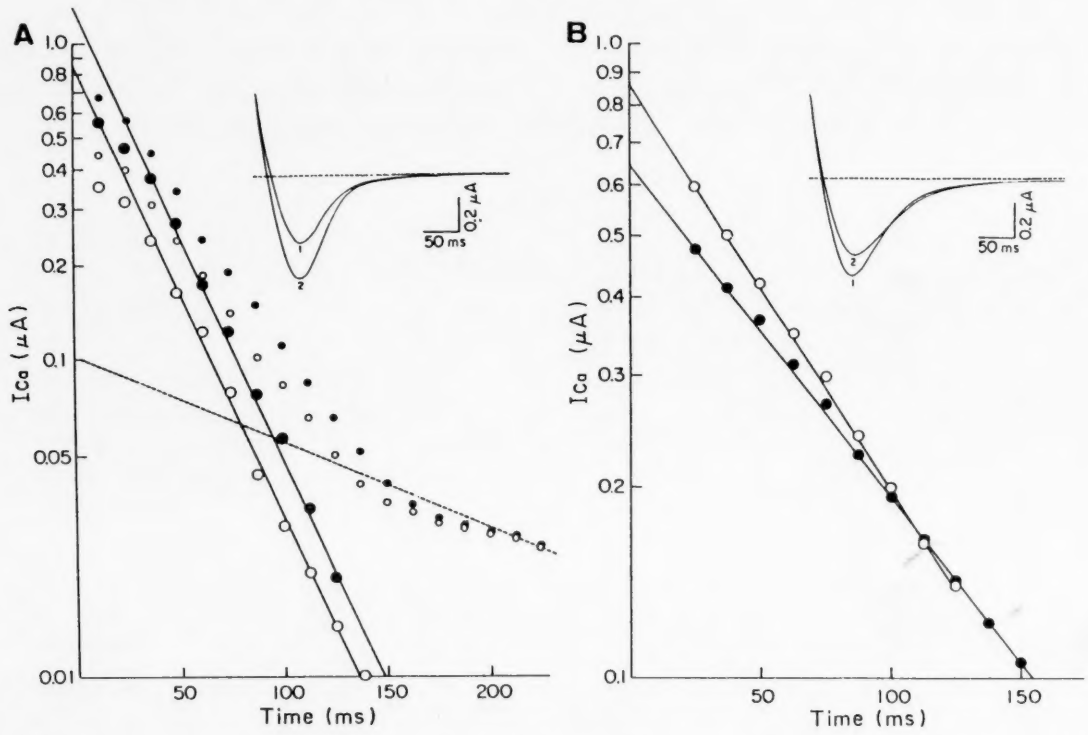

Fig. 5. Rate of inactivation of Ca current obtained before (open circles) and during (filled circles) exposure to two concentrations of ATP. A and B, logarithmic plot of decay of I_{Ca} with 450-ms depolarizing steps from a holding potential of -40 to -21 mV before and during the action of 1.6 μM ATP in each inset. Current records in insets are superimposed with respect to holding current (dotted line) after correction for leakage and residue capacitative currents before (1) and during (2) exposure to ATP. In A, decay follows two exponential terms with time constants of 28 and 160 ms. The time constant of the fast component (large circles) was estimated after subtraction of the slow component from the total current (small circles). In B, only one exponential term is evident, corresponding to the fast phase of decay. A straight line was fitted to the fast phase. The time constant was 65 ms in control and 85 ms in test medium.

items 8 and 9), since no detectable change in $\bar{g}_a$ are seen (table 2, item 7). The rate of activation is slowed by ATP, so that the peak of the action potential is delayed (table 2, item 3). The rate of inactivation is considerably slowed by ATP, and the observed prolongation in the repolarizing phase of the action potential (table 2, item 3) may thus be attributed to this slowing of the rate of inactivation (table 2, item 9). Although a reduction in the K channel conductance (g_K) can produce a results similar to those described, the present data indicate that ATP produces a slight increase or no change in $\bar{g}_K$ (table 2, item 10). Therefore, the possible participation of I_K in the prolongation of the action potential can be excluded.

It thus follows that changes in the amplitude and kinetics of I_{Ca} in response to two different concentrations are primarily responsible for the different observations on action potential, suggesting that different mechanisms may be involved in the ATP-induced effects.

These effects appear quite comparable to those of the α-adrenoceptor agonist known to have a positive inotropic action similar to that of ATP. In the vas deferens muscle, noradrenaline (NA) was found to cause initially an enhancement of I_{Ca} and I_K followed by depression, suggesting a possible dual action of NA on exposure time (Inomata et al., 1989; Mimata and Inomata, 1989). The enhanced effect of lower ATP concentrations thus appears to be virtually the same as the initial effect of NA, while the depressed effect at higher concentrations appears to be somewhat similar to the late effect of NA. However, this enhanced effect of ATP could not be eliminated by phentolamine even at a concentration higher than that necessary to suppress the depolarization induced in the membrane by α-adrenoceptor activation (Wakui and Inomata, 1983). Besides, it was found recently that AMP-PNP, an unhydrolyzable ATP analogue always has the same effect as ATP in the guinea-pig vas deferens muscle (Ji and Inomata, 1990). Therefore, extracellular ATP quite likely acts directly on some receptor (possible P_2-purinoceptor) located at the outer surface of the membrane with other than α-adrenoceptor agonist-like effects.

Although little is known about the transduction mechanism for the ATP-induced changes in I_{Ca} we now observed, metabolism of 3′,5′-cyclic nucleotides or polyphosphoinositide might be involved as generally described for excitatory agonist-induced effects (see review by Reuter, 1983; Berridge, 1984). However, it is currently accepted that extracellular ATP controls cell function by elevating the cytosolic free Ca concentration linked to phosphoinositol (PI) metabolism in non-excitable cells (Dubyak and Young, 1985; Hosoi and Edidin, 1989) or in excitable cells (Phaneuf et al., 1987; Legssyer et al., 1988); that is, the generation of inositol triphosphate (IP_3) may be associated with intracellular Ca mobilization, while the generation of diacylglycerol (DAG) could be associated with an increase in membrane permeability to Ca ions. Whereas there is no direct evidence for ATP effects on Ca channels mediated by alteration in PI breakdown in the vas deferens muscle cells, it was found recently, in cultured vas deferens muscle cells that the Ca influx through the membrane induced by ATP-receptor stimulation was associated with Ins P_4 formation (Hoiting et al., 1990). This result suggests that an enhancement or a depression in I_{Ca} induced by ATP-receptor stimulation such as observed in the present experiments could be accounted for by a mechanism such as just described. That is, the ATP-induced enhancement of I_{Ca} may be due to increased activation of voltage-dependent Ca channels through activation of protein kinase C by DAG, as proposed for muscarinic receptor and adrenoceptor reactions in other smooth muscle cells (Vivaudou et al., 1988; Loirand et al., 1990) or for P_2-purinoceptor reaction in cardiac muscle cells (Alvarez et al., 1990). The ATP-induced depression however appears due to a reduced Ca driving force or to inactivation of Ca channels owing to an increase in cytosolic Ca concentration from opening of the receptor-operated Ca channels and/or to facilitation of the release of Ca from cellular storage sites as observed in other smooth muscles (Den Hertog, 1982; Benham and Tsien, 1987; Friel, 1988).

In conclusion,there appears to be difference in mode of action of extracellularly applied ATP in the vas deferens muscle, depending on its concentration. A low ATP concentration activates voltage-dependent Ca channels through the membrane and a high ATP concentration also stimulates intracellular Ca mobilization.

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References

Alvarez, J.L., K. Mongo, F. Scamps and G. Vassort, 1990, Effects of purinergic stimulation the Ca current in single cardiac cells, Pflügers Arch. 416, 189.

Benham, C.D. and R.W. Tsien, 1987, A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle, Nature 328, 275.

Berridge, M.J., 1984, Inositol triphosphate and diacylglycerol as second messengers, Biochem. J. 220, 345.

Burnstock, G., 1981, Purinergic Receptors (Chapman and Hall, London).

Den Hertog, A., 1982, Calcium and the action of adrenaline, adenosine triphosphate and carbachol of guinea-pig taenia caeci, J. Physiol. 325, 423.

Doroshenko, P.A., P.G. Kostyuk, A.E. Martnyuk, M.D. Kursky and Z.D. Vorobetz, 1984, Intracellular protein kinase and calcium inward currents in perfused neurones of the snail Helix pomatia, Neuroscience 11, 263.

Dubyak, G.R. and M.B.D. Young, 1985, Intracellular Ca^{2+} mobilization activated by extracellular ATP in Ehrlich ascites tumor cells, J. Biol. Chem. 260, 10653.

Flitney, F.W. and J. Singh, 1980, Inotropic responses of the frog ventricle to adenosine triphosphate and related changes in endogenous cyclic nucleotides, J. Physiol. 304, 21.

Friel, D.D., 1988, An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens, J. Physiol. 401, 361.

Goto, M., A. Yatani and Y. Tsuda, 1977, An analysis of the action of ATP and related compound on membrane current and tension components in bull-frog atrial muscle, Jap. J Physiol. 27, 81.

Hoiting, B., A. Molleman, A. Nelemans and A. Den Hertog, 1990, P_2-purinoceptor-activated membrane currents and inosito tetrakisphosphate formation are blocked by suramin, Eur. J. Pharmacol. 181, 127.

Hosoi, H. and M. Edidin, 1989, Exogenous ATP and other nucleoside phosphate epidermal growth factor receptors of A-431 epidermoid carcinoma cells, Proc. Natl. Acad. Sci. U.S.A. 86, 4501.

Hume, R.I. and S.A. Thomas, 1988, Multiple actions of adenosine 5′-triphosphate on chick skeletal muscle, J. Physiol. 406, 503.

Inomata, H. and C.Y. Kao, 1976, Ionic currents in the guinea-pig taenia coli, J. Physiol. 255, 443.

Inomata, H. and M. Wakui, 1984, Behaviour of Ca^{2+} current mediating the inotropic effect of ATP in smooth muscle of the guinea-pig vas deferens, J. Muscle Res. Cell Mot. 5, 476.

Inomata, H. and C.Y. Kao, 1985, Actions of Ba^{++} on ionic currents of the guinea-pig taenia coli, J. Pharmacol. Exp. Ther. 233, 112.

Inomata, H., T. Mimata, M. Wakui and K.Y. Zhang, 1989, Depression of voltage-dependent Ca^{2+} current by noradrenaline in the guinea-pig vas deferens in tetraethylammonium medium, Jap. J. Physiol. 39, 455.

Irisawa, H. and S. Kokubun, 1983, Modulation by intracellular ATP and cyclic AMP of the slow inward current in isolated single ventricular cells of the guinea-pig, J. Physiol. 338, 321.

Ji, J.J. and H. Inomata, 1990, The effects of non-hydrolyzable analogue of ATP on current- and voltage-clamped muscle cells of the guinea-pig vas deferens, Jap. J. Physiol. 40, S-257.

Kao, C.Y. and H. Inomata, 1986, Double Sucrose-Gap Voltage-Clamp Technique-Possibilities and Limitations, in: Prodeedings of Smooth Muscle Function Symposium of the 30th IUPS, p. 28 (abstr.).

Klöckner, U. and G. Isenberg, 1985, Calcium current of cesium loaded isolated smooth muscle cells (urinary bladder of the guinea-pig), Pflügers Arch. 405, 340.

Legssyer, A., J. Poggioli, D. Renard and G. Vassort, 1988, ATP and other adenine compounds increase mechanical activity and inositol triphosphate production in rat heart, J. Physiol. 401, 185.

Loirand, G., P. Pacaud, C. Mironneau and J. Mironneau, 1990, GTP-binding proteins mediate noradrenaline effects on calcium and chloride currents in rat portal vein, J. Physiol. 428, 517.

Mimata, T. and H. Inomata, 1989, Effect of noradrenaline on current- and voltage-clamped muscle cells of the guinea-pig vas deferens in normal Krebs solution, Eur. J. Pharmacol. 163, 181.

Molleman, A., A. Nelemans and A. Den Hertog, 1989, P_2-purinoceptor-mediated membrane currents in DDT_1 MF-2 smooth muscle cells, Eur. J. Pharmacol. 169, 167.

Nakazawa, K. and N. Matsuki, 1987, Adenosine triphosphate-activated inward current in isolated smooth muscle cells from rat vas deferens, Pflügers Arch. 409, 644.

Ohya, Y., K. Kitamura and H. Kuriyama, 1987, Modulation of ionic currents in smooth muscle balls of the rabbit intestine by intracellular perfused ATP and cyclic AMP, Pflügers Arch. 408, 466.

Ohya, Y. and N. Sperelakis, 1989, ATP regulation of the slow calcium channels in vascular smooth muscle cells of the guinea-pig mesenteric artery, Circ. Res. 64, 145.

Phaneuf, S., P. Berta, J. Casanova and J.C. Cavadore, 1987, ATP stimulates inositol phosphate accumulation and calcium mobilization in a primary culture of rat aortic myocytes, Biochem. Biophys. Res. Commun. 143, 454.

Reuter, H., 1983, Calcium channel modulation by neurotransmitters, enzymes and drugs, Nature 301, 569,

Sneddon, P. and D.P. Westfall, 1984, Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitter in the guinea-pig vas deferens, J. Physiol. 347, 561.

Vivaudou, M.B., L.H. Clapp, J.V. Walsh and J.J. Singer, 1988, Regulation of one type of Ca^{2+} current in smooth muscle cells by diacylglycerol and acetylcholine, FASEB J. 2, 2497.

Wakui, M. and H. Inomata, 1983, Effect of cobalt on electrical and mechanical responses to exogenous noradrenaline and to field stimulation in the guinea-pig vas deferens, Jap. J. Physiol. 33, 1057.

Wakui, M. and H. Inomata, 1985, Evidence for an increase in membrane conductance during adenosine triphosphate-induced depolarization in the guinea-pig vas deferens, Pflügers Arch. 403, 112.

Yamagata, H., N. Hoshimiya and H. Inomata, 1983, Voltage-clamp processor system, IEEE Trans. Biomed. Eng. 30, 103.

Yatani, A., N. Tsuda, N. Akaike and A.M. Brown, 1982, Nanomolar concentrations of extracellular ATP activates membrane Ca channels in snails neurones, Nature 296, 169.

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CCK_A receptor antagonism inhibits mechanisms underlying CCK-8-stimulated insulin release in isolated rat islets

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The influence of the cholecystokinin $(CCK)_A$ receptor antagonist, L-364,718, on β-cell activation was examined in isolated perifused prelabelled rat islets. Insulin secretion and ^3H efflux from myo-[2-^{3}H]inositol-prelabelled islets (reflecting phosphoinositide hydrolysis) stimulated by CCK-8 (100 nM) were both inhibited by L-364,718, partially at 1 nM and totally at 10 nM. $^{45}Ca^{2+}$ efflux from prelabelled islets was markedly stimulated by CCK-8. This stimulation was inhibited equally by 1 and 10 nM L-364,718. CCK-8 stimulated the $^{86}Rb^+$ efflux (reflecting K^+ movements) from prelabelled islets, which probably reflects an indirect effect of CCK-8 due to opening of Ca^{2+}-activated K^+ channels. This $^{86}Rb^+$ efflux was inhibited by L-364,718 at 10 nM but not affected by L-364,718 at 1 nM. It is concluded that insulin secretion, phosphoinositide hydrolysis, Ca^{2+} and K^+ movements stimulated by CCK-8 in isolated islets are all events mediated by CCK_A receptors. The L-364,718-induced inhibition of phosphoinositide hydrolysis was most closely correlated to the inhibition of insulin secretion. This suggests that induction of cellular events activated through stimulation of phosphoinositide hydrolysis is a major mechanism underlying CCK-8-stimulated insulin secretion.

CCK-8 (cholecystokinin-8); Insulin secretion; Islets; CCK_A receptor antagonists; L-364,718; Ca^{2+}; K^+; Phosphoinositide hydrolysis

1. Introduction

The octapeptide of cholecystokinin (CCK-8) stimulates insulin secretion (Ahrén and Lundquist, 1981), and specific CCK receptors have been demonstrated in the islets of Langerhans (Sakamoto et al., 1985; Verspohl et al., 1986). The exact cellular mechanism underlying the stimulatory effect of the peptide is not yet known, although CCK-8 has been shown to stimulate phosphoinositide (PI) hydrolysis (Zawalich et al., 1987) and to affect the cellular handling of Ca^{2+} and K^+ in isolated rat islets (Verspohl et al., 1987; Zawalich et al., 1987). It is known that the CCK receptor antagonist, L-364,718 is selective for peripheral CCK (CCK_A) receptors (Chang and Lotti, 1986; Evans et al., 1986). Previously, L-364,718 has been shown to selectively inhibit CCK-8-stimulated insulin secretion in the mouse (Karlsson and Ahrén, 1989) as well as to inhibit CCK-8-stimulated PI-hydrolysis in isolated rat islets (Zawalich et al., 1988). However, the effects of the antagonist on other events that might underlie CCK-8-induced insulin secretion are not known. We therefore investigated the effects of the CCK_A receptor antagonist, L-364,718, on CCK-8-stimulated $^{45}Ca^{2+}$ efflux and $^{86}Rb^+$ efflux (which reflect Ca^{2+} and K^+ movements) as well as on CCK-8-stimulated insulin secretion and PI hydrolysis in prelabelled, perifused rat islets of Langerhans.

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2. Materials and methods

2.1. Animals

The experiments were performed with pancreatic islets isolated from male Sprague–Dawley rats (ALAB, Stockholm, Sweden) weighing 200–250 g. The animals had free access to a standard pellet diet (Astra-Ewos, Södertälje, Sweden) and tap water before the experiments.

2.2. Isolation of islets

Pancreatic islets were isolated by collagenase digestion (type V, Sigma Chemical Co., St Louis, Mo., U.S.A) (1.3 mg/ml tissue) in Hank's balanced salts solution (Sigma Chemical Co., St Louis, Mo., USA)

(Lacy and Kostianovsky, 1967). After isolation, the islets were cultured over night in a RPMI 1640 medium supplemented with 10% fetal calf serum, 200 mM L-glutamine, 100 IU/ml penicillin G, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B at 37°C in an atmosphere with 5% CO_2.

2.3. Perifusion experiments

After overnight culture, the islets were washed in a modified KRB Hepes buffer supplemented with 0.02% human serum albumin and 3.3 mM glucose and consisting of 114 mM NaCl, 4.4 mM KCl, 1 mM $MgSO_4$, 29.5 mM $NaHCO_3$, 1.28 mM $CaCl_2$ and 10 mM HEPES. Thereafter, the islets were prelabelled with $^{45}Ca^{2+}$, $^{86}Rb^{+}$ or myo-[2-^{3}H]inositol (DuPont Scandinavia A.B., Stockholm, Sweden). For the $^{45}Ca^{2+}$ experiments, 160 islets were loaded for 90 min in 750 μl of KRB buffer (16.7 mM glucose) to which was added 50 μl $^{45}CaCl_2$ (50–100 μCi) from a stock solution (specific activity, 26–37 mCi/mg). In the $^{86}Rb^{+}$ experiments, 160 islets were loaded for 90 min in 1000 μl of KRB buffer (3.3 mM glucose) supplemented with ^{86}RbCl (40–170 μCi), added from a stock solution with an initial specific activity of 1.6–6.4 mCi/mg. The subsequent $^{86}Rb^{+}$ efflux reflects K^{+} movements. In the experiments on PI hydrolysis, 160 islets were labelled for 120 min in 800 μl of KRB buffer with 3.3 mM glucose containing 25 μCi of myo-[2-^{3}H]inositol added from a stock solution (specific activity, 20 Ci/mmol). The subsequent release of ^{3}H indirectly reflects PI hydrolysis (Clements and Rothen, 1976; Mathias et al., 1985; Zawalich and Zawalich, 1988). After labelling, groups of 30-40 islets were perifused at a flow rate of 0.1 ml/min (Skoglund et al., 1988). The perifusion medium was continously gassed with 95% O_2–5% CO_2. The perifusate was collected in 2-min samples. A 50 μl aliquot was removed from each sample for determination of insulin immunoreactivity, and 100 μl was removed and added to 5 ml scintillation fluid (Ready Safe ™, Beckman Instruments Inc., Fullerton, CA, U.S.A.) for liquid scintillation counting (Packard Instrument Inc., Downers Groove, Ill., U.S.A.). When ^{3}H was to be counted, 0.5 ml of 0.5 M HCl was added to the scintillation fluid. At the end of each perifusion experiment, the islets were collected and the radioactivity remaining in the islets was determined.

2.4. Incubation experiments

After overnight culture the islets were washed in the modified KRB Hepes buffer and preincubated for 45 min. Thereafter, single islets were incubated for 60 min in 0.1 ml of the KRB medium containing 8.3 mM glucose with or without the addition of CCK-8 (1–1000 nM) as previously described (Skoglund et al., 1988). After incubation, 50 μl of the incubation medium was removed from each chamber for determination of its insulin immunoreactivity.

2.5. Insulin analysis

The concentration of insulin was determined with a radioimmunoassay, using a guinea pig anti-porcine insulin antibody (Milab AB, Malmö, Sweden), ^{125}I-labelled porcine insulin as a tracer and porcine insulin as a standard (Novo Res, Bagsvaerd, Denmark). Bound and free radioactivity were separated with the dextran-coated charcoal technique (Herbert et al., 1965).

2.6. Drugs

Cholecystokinin-8 (sulfated form) was from Sigma Chemical Co. (St Louis, Mo., U.S.A). L-364,718 (3s(–)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)1H-indole-2-carboxamide) was kindly supplied by Dr V. Lotti, Merck Sharp and Dohme Research Laboratories (West Point, Pa, U.S.A). L-364,718 was initially dissolved in dimethyl sulfoxide and was thereafter further diluted in the modified KRB Hepes medium. The concentration of dimethylsulfoxide did not exceed 0.01%.

2.7. Statistics

The results are given as means ± S.E.M. The perifusion data are presented as the fractional outflow of $^{45}Ca^{2+}$, $^{86}Rb^{+}$ or ^{3}H from islets as calculated for each time point. In the perifusion experiments the degree of statistical significance was calculated by two-way analysis of variance (ANOVA). A probability level of a random difference of $P < 0.01$ was considered significant. Student's t-test for unpaired data was used for statistical evaluation in the incubation experiments and the probability levels of a random difference are given.

3. Results

3.1. Effects of L-364,718 on CCK-8-stimulated insulin secretion (fig. 1)

The concentration response relationship (1–1000 nM) for CCK-8-stimulated insulin release from isolated statically incubated rat islets is shown in table 1. From these results, a dose level of 100 nM was selected for the following perifusion studies. CCK-8 (100 nM) induced a prompt and sustained insulin secretion in perifused islets. The insulin secretion remained elevated throughout the perifusion period. This increase

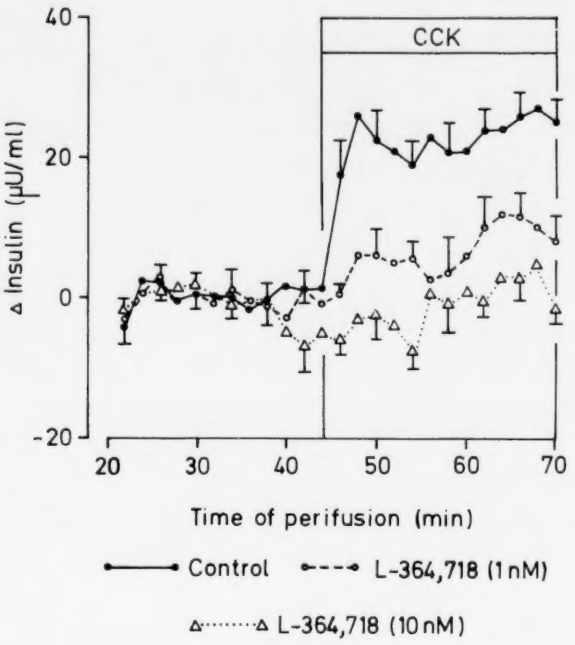

Fig. 1. Effects of L-364,718 on CCK-8-stimulated insulin secretion from perifused rat islets of Langerhans. (●) Represents control columns to which CCK-8 alone was added to an ongoing perifusion with 8.3 mM glucose. L-364,718 (1 nM ○), (10 nM △) was introduced in the perifusion medium 14 min before the introduction of a medium containing CCK-8 (100 nM) + L-364,718 (1 or 10 nM). The values are given as the increase in insulin levels in the perifusate as calculated from basal levels of insulin between min 22 and 26. Means ± S.E.M. are shown. n = 8–10.

in insulin secretion was partially supressed by L-364,718 at 1 nM and was totally inhibited by the antagonist at 10 nM.

3.2. Effects of L-364,718 on CCK-8-stimulated ^3H efflux from myo-[2-^3H]inositol-prelabelled islets (fig. 2)

CCK-8 (100 nM) markedly stimulated ^3H efflux from myo-[2-^3H]inositol-prelabelled islets. The increase started 6 min after the addition of CCK-8 and rose gradually throughout the perifusion period. The stimulation was partially inhibited by L-364,718 at 1 nM and completely abolished by L-364,718 at 10 nM.

TABLE 1

Concentration–response relationship for CCK-8-stimulated insulin release. Isolated rat islets of Langerhans were incubated for 60 min in a KRB buffer supplemented with 8.3 mM glucose with and without the addition of CCK-8 at different dose levels. Means ± S.E.M. are shown. n = 15–16. a and b indicate probability levels of random difference in insulin release from control islets versus islets incubated with CCK-8.

CCK-8 (nM)	Insulin (μU/ml)
–	44 ± 5
1	47 ± 6
10	58 ± 4 [a]
100	78 ± 3 [b]
1000	64 ± 5 [b]

[a] $P < 0.05$; [b] $P < 0.001$.

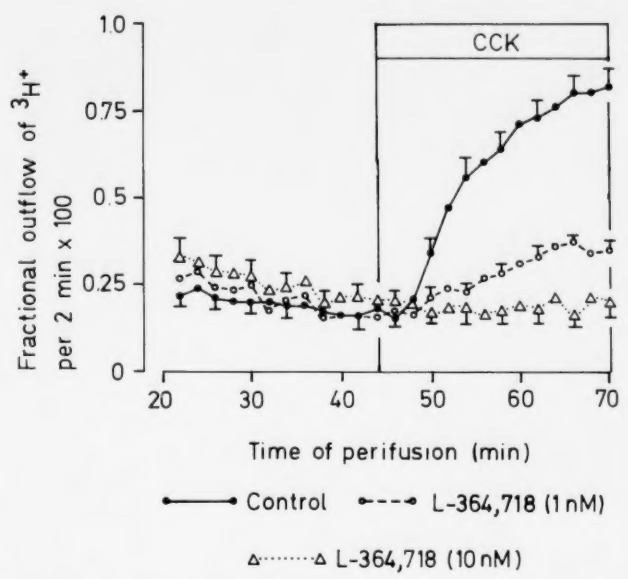

Fig. 2. Effects of L-364,718 on CCK-8-stimulated ^3H efflux from myo-[2-^3H]inositol-prelabelled perifused rat islets of Langerhans. (●) Represents control columns to which CCK-8 alone was added to an ongoing perifusion with 8.3 mM glucose. L-364,718 (1 nM ○) (10 nM △) was introduced to the medium 14 min before the introduction of a medium containing CCK-8 (100 nM) + L-364,718 (1 or 10 nM). The values are given as the fractional efflux of ^3H. Means ± S.E.M. are shown. n = 4–8.

3.3. Effects of L-364,718 on CCK-8-stimulated $^{45}Ca^{2+}$ efflux (fig. 3)

CCK-8 (100 nM) induced a rapid increase in $^{45}Ca^{2+}$ efflux with a peak at 4 min. Thereafter there was a

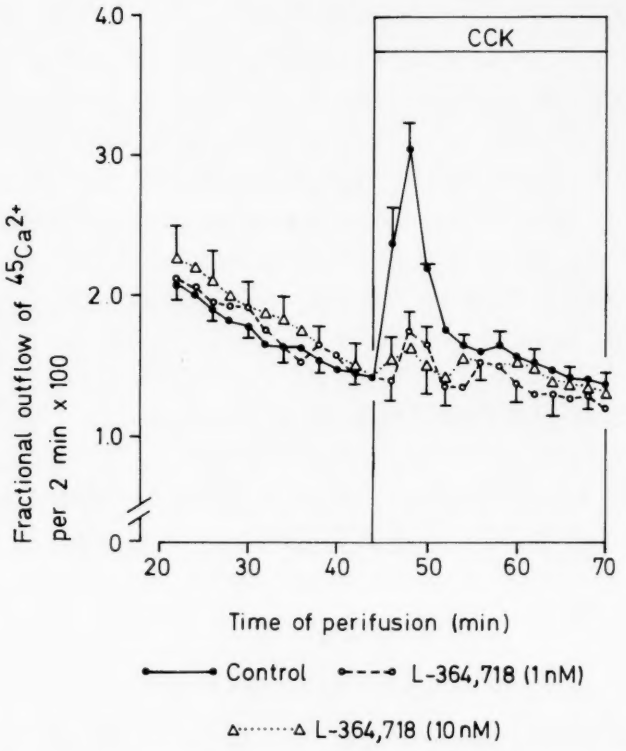

Fig. 3. Effects of L-364,718 on CCK-8-stimulated $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-prelabelled perifused rat islets of Langerhans. (●) Represents control columns to which CCK-8 alone was added to an ongoing perifusion with 8.3 mM glucose. L-364,718 (1 nM ○) (10 nM △) was introduced to the perifusion medium 14 min before the introduction of a medium containing CCK-8 (100 nM) + L-364,718 (1 or 10 nM). The values are given as the fractional efflux of $^{45}Ca^{2+}$. Means ± S.E.M. are shown. n = 4–8.

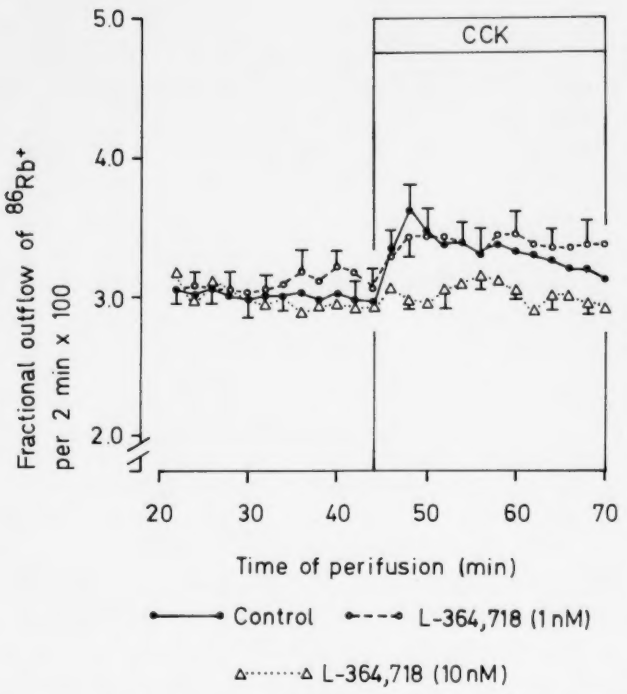

Fig. 4. Effects of L-364,718 on CCK-8-stimulated $^{86}Rb^+$ efflux from $^{86}Rb^+$-prelabelled perifused rat islets of Langerhans. (●) Represents control columns to which CCK-8 alone was added to an ongoing perifusion with 8.3 mM glucose. L-364,718 (1 nM ○) (10 nM △) was introduced to the perifusion medium 14 min before the introduction of a medium containing CCK-8 (100 nM) + L-364,718 (1 or 10 nM). The values are given as the fractional efflux of $^{86}Rb^+$. Means ± S.E.M are shown. n = 4–8.

sustained efflux at a level above that observed before the introduction of the peptide. L-364,718 markedly inhibited the CCK-8-induced $^{45}Ca^{2+}$ efflux at both 1 and 10 nM.

3.4. Effects of L-364,718 on CCK-8-stimulated $^{86}Rb^+$ efflux (fig. 4)

CCK-8 (100 nM) stimulated a rapid and sustained efflux of $^{86}Rb^+$ from prelabelled islets. L-364,718 at 10 nM totally abolished this CCK-8-induced increase in $^{86}Rb^+$ efflux. The lower dose level of the antagonist (1 nM) was, however, without effect.

3.5. Effects of L-364,718 with 8.3 mM glucose (figs. 1–4)

L-364,718 by itself, when added to the ongoing glucose perifusion 14 min before the addition of CCK-8 to the medium, did not significantly influence insulin release, $^{45}Ca^{2+}$ efflux, ^3H efflux or $^{86}Rb^+$ efflux.

4. Discussion

We here confirmed the results from previous studies that CCK-8 stimulates insulin secretion from rat islets (Zawalich et al., 1986; Verspohl et al., 1987) as well as $^{45}Ca^{2+}$ efflux from islets prelabelled with $^{45}Ca^{2+}$ (Zawalich et al., 1987). These effects could reflect the fact that the insulinotropic effect of CCK-8 is due to an increased Ca^{2+} influx. Furthermore, we also confirmed that CCK-8 stimulates the ^3H efflux from myo-[2-^3H]-inositol-prelabelled islets (Zawalich et al., 1987), which is indirect evidence for stimulation of PI hydrolysis (Clements and Rothen, 1976; Mathias et al., 1985; Zawalich and Zawalich, 1988). Therefore, inositol-1,4,5-trisphosphate (IP_3), generated by CCK-8-stimulated PI hydrolysis (Florholmen et al., 1989), might mobilize intracellularly stored Ca^{2+}. Thus, this mechanism also might contribute to CCK-8-stimulated insulin release. The net effect of increased Ca^{2+} influx and release of intracellularly stored Ca^{2+} would be an increased intracellular concentration of Ca^{2+}, which is known to be an important process in the mechanism of insulin release (Prentki and Matschinsky, 1987). However, the relative contribution of Ca^{2+} influx relative to mobilization of intracellularly stored Ca^{2+}, to the $^{45}Ca^{2+}$ efflux stimulated by CCK-8 cannot be deduced from the present study, but has to await demonstration in studies performed in the absence of extracellular Ca^{2+}.

It was verified that the CCK receptor antagonist, L-364,718, dose dependently inhibits CCK-8-induced insulin secretion in rat islets (Zawalich et al., 1988). This suggests that the beta cells are equipped with CCK receptors of the CCK_A subtype, since L-364,718 is known to be a selective CCK_A receptor antagonist (Chang and Lotti, 1986). The inhibition of insulin secretion by L-364,718 was accompanied by the inhibition of both $^{45}Ca^{2+}$ and ^3H efflux from prelabelled islets. This shows that these two CCK-8-induced islet events are also dependent on intact CCK_A receptors. There was a parallel inhibition by L-364,718 of insulin secretion and ^3H efflux in that, at 1 nM, the antagonist inhibited both processes only partially whereas at 10 nM, insulin secretion and ^3H efflux were completely abolished. In contrast, the CCK-8-stimulated $^{45}Ca^{2+}$ efflux was almost equally suppressed by L-364,718 at both 1 and 10 nM. This suggests that the processes underlying stimulation of $^{45}Ca^{2+}$ efflux, i.e., stimulation of Ca^{2+} uptake and/or mobilization of intracellularly stored Ca^{2+}, might not be the major mechanisms for the insulin secretory action of CCK-8. Our results may suggest instead that PI hydrolysis, having an impact on cellular events other than Ca^{2+} fluxes, is a more important pathway underlying CCK-8-induced insulin secretion. Such a mechanism could be activation of protein kinase C (PKC) by diacylglycerol (Prentki and Matschinsky, 1987) which is known to be generated by PI hydrolysis (Berridge, 1984). In fact, we have previously shown that CCK-8-stimulated insulin secretion is partially dependent on the PKC pathway (Karlsson and Ahrén, 1991).

CCK-8 has been shown to stimulate $^{86}Rb^+$ efflux from prelabelled islets, which probably reflects an indirect effect of CCK-8 exerted through Ca^{2+}-dependent

K^+ channels activated by an increased concentration of intracellular Ca^{2+} (Verspohl et al., 1987). This effect of CCK-8 on $^{86}Rb^+$ efflux differs from the effect of glucose, which is known to decrease $^{86}Rb^+$ permeability in islets (Sehlin and Täljedal, 1975). We found that the CCK-8-induced stimulation of $^{86}Rb^+$ efflux was inhibited by 10 nM, but not by 1 nM, L-364,718. Thus, insulin secretion was markedly inhibited by L-364,718 at 1 nM whereas the $^{86}Rb^+$ efflux was not affected at this concentration of the antagonist. This indicates that the presumably very small increase in intracellular Ca^{2+} levels that develops in the presence of 1 nM L-364,718 (since PI hydrolysis is not completely abolished) is sufficient to maximally activate the Ca^{2+}-dependent K^+ channels. The magnitude of this increase however, seems insufficient for a maximal activation of insulin secretion. An alternative, though less likely, explanation is that these K^+ channels are regulated by CCK receptors other than those involved with PI hydrolysis and that these CCK receptors have a low sensitivity to L-364,718.

In conclusion, CCK-8-induced stimulation of insulin secretion, PI hydrolysis, $^{45}Ca^{2+}$ and $^{86}Rb^+$ efflux from prelabelled rat islets are all events mediated by CCK_A receptors since they are all sensitive to inhibition by L-364,718. Our present results also showed that the L-364,718-induced inhibition of CCK-8-stimulated PI hydrolysis is the process that is most closely correlated to the concomitant inhibition of insulin secretion. This, in turn, suggests that PI hydrolysis is a major mechanism underlying the insulin secretion stimulated by CCK-8.

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References

Ahrén, B. and I. Lundquist, 1981, Effects of two cholecystokinin variants, CCK-39 and CCK-8, on basal and stimulated insulin secretion, Acta Diabetol. Lat. 18, 345.

Berridge, M.J., 1984, Inositol trisphosphate and diacylglycerol as second messengers, Biochem. J. 220, 345.

Chang, R.S.L. and V.J. Lotti, 1986, Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist, Proc. Natl. Acad. Sci. U.S.A. 83, 4923.

Clements, R.S., Jr. and W.B. Rothen, 1976, Phosphoinositide metabolism and insulin secretion from isolated rat pancreatic islets, J. Clin. Invest. 57, 684.

Evans, B.E., M.G. Bock, K.E. Rittle, R.M. Dipardo, W.L. Whitter, D.F. Veber, P.S. Anderson and R.M. Freidinger, 1986, Design of potent, orally effective, nonpeptidal antagonists of the peptide hormone cholecystokinin, Proc. Natl. Acad. Sci. U.S.A. 83, 4918.

Florholmen, J., D. Malm, B. Vonen and P.G. Burhol, 1989, Effect of cholecystokinin on the accumulation of inositol phosphates in isolated pancreatic islets, Am. J. Physiol. 257, G865.

Herbert, V., K.S. Lau, C.W. Gottlieb and S.J. Bleicher, 1965, Coated charcoal immunoassay of insulin, J. Clin. Endocrinol. Metab. 25, 1875.

Karlsson, S. and B. Ahrén, 1989, Effects of threee different cholecystokinin receptor antagonists on basal and stimulated insulin and glucagon secretion in mice, Acta Physiol. Scand. 135, 271.

Karlsson, S. and B. Ahrén, 1991, Cholecystokinin-stimulated insulin secretion and protein kinase C in rat pancreatic islets, Acta Physiol. Scand. 142, 397.

Lacy, P.E. and M. Kostianovsky, 1967, Method for the isolation of intact islets of Langerhans from the rat pancreas, Diabetes 16, 35.

Mathias, P.C.F., L. Best and W.J. Malaisse, 1985, Stimulation by glucose and carbamylcholine of phospholipase C in pancreatic islets, Cell. Biochem. Funct. 3, 173.

Prentki, M. and F.M. Matschinsky, 1987, Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion, Physiol. Rev. 67, 1185.

Sakamoto, C., I.D. Goldfine, E. Roach and J.A. Williams, 1985, Localization of saturable CCK binding sites in rat pancreatic islets by light and electron microscope autoradiography, Diabetes 34, 390.

Sehlin, J. and I.-B. Täljedal, 1975, Glucose-induced decrease in Rb^+ permeability in pancreatic β cells, Nature 253, 635.

Skoglund, G., I. Lundquist and B. Ahrén, 1988, Selective α_2-adrenoceptor activation by clonidine: effects on $^{45}Ca^{2+}$-efflux and insulin secretion from isolated rat islets, Acta Physol. Scand. 132, 289.

Verspohl, E.J., H.P.T. Ammon, J.A. Williams and I.D. Goldfine, 1986, Evidence that cholecystokinin interacts with specific receptors and regulates insulin release in isolated rat islets of Langerhans, Diabetes 35, 38.

Verspohl, E.J., I. Breuning, H.P.T. Ammon and M. Mark, 1987, Significance of Ca^{2+}, Rb^+ fluxes, of cAMP and cGMP for the CCK_8-modulated insulin release, Reg. Pept. 17, 229.

Zawalich, W.S., S.B. Cote and V.A. Diaz, 1986, Influence of cholecystokinin on insulin output from isolated perifused pancreatic islets, Endocrinology 119, 616.

Zawalich, W.S., V.A. Diaz and K.C. Zawalich, 1988, Stimulatory effects of cholecystokinin on isolated perifused islets inhibited by potent and specific antagonist L 364718, Diabetes 37, 1432.

Zawalich, W., N. Takuwa, Y. Takuwa, A. Diaz and H. Rasmussen, 1987, Interactions of cholecystokinin and glucose in rat pancreatic islets, Diabetes 36, 426.

Zawalich, W.S. and K.C. Zawalich, 1988, Phosphoinositide hydrolysis and insulin release from isolated perifused rat islets. Studies with glucose, Diabetes 37, 1294.

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Protective effects of R 56 865 against ischemic damage in the isolated rabbit heart

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We examined the role of calcium in the pathogenesis of ischemic cardiac cell death in the isolated working rabbit heart subjected to normothermic global ischemia followed by reperfusion. Apart from measurements of cardiodynamic function and ultrastructural examination, we also used a cytochemical procedure to localize exchangeable calcium pools at the ultrastructural level. The effects of verapamil (1.5×10^{-8} M, 3×10^{-8} M) (high affinity for L-type calcium channels) were compared with those of R 56 865 (4×10^{-7} M) (Ca^{2+} overload blocker with low affinity for the L-type calcium channels). A severe depression of cardiac function was observed after solvent or verapamil pretreatment and 25 min of ischemia followed by reperfusion. R 56 865 treatment resulted in a significantly improved postischemic recovery when compared to solvent and verapamil treatment groups. The ultrastructural and cytochemical results corroborated the hemodynamic findings. In solvent and verapamil-treated hearts, irreversible damage was observed mainly in mid- and endocardial areas. Ultrastructural changes were accompanied by shifts in calcium localization: i.e. loss of sarcolemmal calcium binding capacity, accumulation of calcium precipitate in the mitochondria. In the R 56 865 treatment group, damage was limited to some cells scattered in the midcardial areas. In conclusion, R 56 865, which has little affinity for the slow channels was highly effective in protecting against ischemic damage, indicating that, in this experimental set-up, the calcium responsible for cellular Ca^{2+} overload is not entering via L-type calcium channels.

R 56 865; Ca^{2+} overload; Ischemia; Reperfusion; Heart

1. Introduction

The possibility to protect myocardial cells against ischemia has received considerable attention. In order to prevent or decrease myocardial cell necrosis, more knowledge of the events leading to irreversible myocardial cell death must be acquired. Much attention has been focused on the central role of calcium ions in mediating ischemic cell death (Poole Wilson et al., 1984; Cheung et al., 1986; Katz and Reuter, 1979). Indeed, an increase in intracellular free calcium activates calcium-dependent phospholipases, resulting in the breakdown of cell membranes and the loss of ion gradients (Chien et al., 1978). Mitochondria will accumulate large amounts of calcium at the expense of ATP production (Nayler et al., 1988). Important enzymes (e.g. proteases and ATPases) that are regulated by calcium are activated, and their activation will lead to denaturation of proteins and an enhanced consumption of the already sparse ATP pool.

An indirect approach for studying the role of calcium involves the use of different classes of calcium antagonists to examine the relative importance of the different pathways of calcium entry into the cell. In our study we compared the effects of verapamil, which has high affinity for the L-type calcium channel, with those of R 56 865, which has little affinity for the slow calcium channel (Carmeliet and Tytgat, 1991; Himmel et al., 1990). R 56 865 (N-(1-(4-(4-fluoro-phenoxy)butyl)-4-piperidinyl)-N-methyl-2-benzothiazolamine), Janssen Research Foundation, Belgium) has been shown in previous studies to protect against ouabain toxicity (Vollmer et al., 1987; Schneider et al., 1987; Finet et al., 1989) and against functional defects elicited by ischemia or hypoxia (Garner et al., 1989, 1990; Borgers and Ver Donck, 1990; Schleuffer et al., 1990). In addition, we used the cytochemical phosphate pyroantimonate technique to localize exchangeable calcium pools at the subcellular level (Borgers et al. 1984). This method allows different intracellular calcium pools to be localized before and after ischemia.

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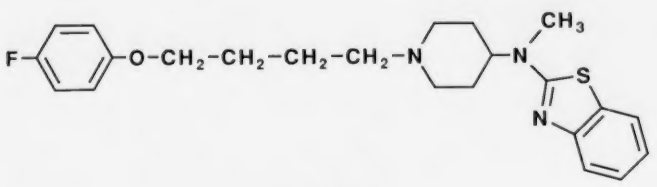
2. Materials and methods

2.1. Surgical procedure and perfusion technique

The perfusion system used has been described in detail previously (Lu et al., 1989). New Zealand rabbits of either sex weighing 2–2.5 kg were used. After premedication with hypnorm (10 mg fluanisone/0.2 mg fentanyl per ml, 0.4 ml/kg i.m.), anesthesia with Nembutal (5 $mg \cdot kg^{-1}$) and tracheotomy, the lungs were ventilated with 50/50 air-oxygen, using a Bird Mark 7 respirator. A thoracotomy was performed and heparin (5000 U.I.) and scopolamine (1 $mg \cdot kg^{-1}$) were injected i.v. The hearts were quickly removed from the thorax, immersed in ice-cold Krebs-Henseleit-bicarbonate buffer (KH in mM: NaCl 118; KCl 14.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; pH 7.4) and lung and fat tissue were removed. The aorta was cannulated, perfused with 20 ml ice-cold KH and attached to the perfusion system where retrograde perfusion was started. One cannula was placed in the pulmonary artery to measure coronary flow (CF) and another in the left atrium for the measurement of left atrial pressure (LAP). Pulmonary veins were ligated. Left ventricular pressure (LVP) and its first derivative ($LVdp/dt_{max}$ and $LVdp/dt_{min}$) were measured via a Millar tip manometer catheter inserted into the left ventricle via the apex and connected to a Statham PL3DM pressure transducer.

2.2. Experimental protocol

The experimental protocol is shown in fig. 1. The hearts were equilibrated for 30 min by retrograde perfusion of the coronary arteries via the aorta (Langendorff, 1895). The system was then switched to the working condition with a constant preload of 10 mm Hg and an afterload of 50 mm Hg. Hemodynamic parameters including heart rate (HR), systolic and diastolic aortic pressure (APs and APd, respectively), LVP, $LVdp/dt_{max}$ and min and LAP were recorded every 10 min on a four-channel recorder. Coronary flow and aortic flow (AF) were measured by separately collecting the total outflows during 1 min. Cardiac output (CO) was calculated by adding coronary and aortic flow values. Fifteen minutes after switching the system to the working heart condition, solvent (β-hydroxypropylcyclodextrine 10%) or drug was added for the next 30 min. The heart was subjected to global



R 56865

Fig. 2. Chemical structure of R 56 865.

normothermic no-flow ischemia by clamping the left atrial and aortic cannulas and by immersing the heart in a chamber filled with non-oxygenated perfusate at 37 °C. After ischemic periods of 20, 25, 30 min followed by 10 min of Langendorff perfusion, the system was switched to the working state again. During the reperfusion period, drug or solvent remained present. The values obtained at the end of 15 min working heart condition (pre-ischemia) were compared with those at the end of 45 min working heart condition (pre-ischemia) in order to assess the effect of solvent and drugs. Values at the end of the experiment were also compared with those at the end of 45-min working heart condition (pre-ischemia) to evaluate the degree of postischemic recovery. A control group (n = 7, no ischemia induced) was examined in order to test the stability of the system. The four groups investigated were solvent, R 56 865 $4 \cdot 10^{-7}$ M, verapamil $1.5 \cdot 10^{-8}$ M and verapamil $3 \cdot 10^{-8}$ M (n = 7 in each group). The chemical structure of R 56 865 is presented in fig. 2.

2.3. Ultrastructure and calcium cytochemistry

The heart was fixed by retrograde perfusion (20 ml/min for 5 min; room temperature) with 3% glutaraldehyde supplemented with 90 mM potassium phosphate and 1.4% sucrose (pH 7.4). Blocks (2 mm thick) were cut out of the left ventricle from four different slices (from apex to base) and immersed in the fixative for at least 2 h at room temperature. After fixation, the hearts were processed for calcium localization and morphology as described (Borgers et al., 1984).

The same was done for five hearts from the solvent- and drug-treated groups.

Semithick sections for light microscopy were stained with toluidine blue. Ultrathin sections for electron microscopy were examined either unstained or counterstained with uranium acetate and lead citrate.

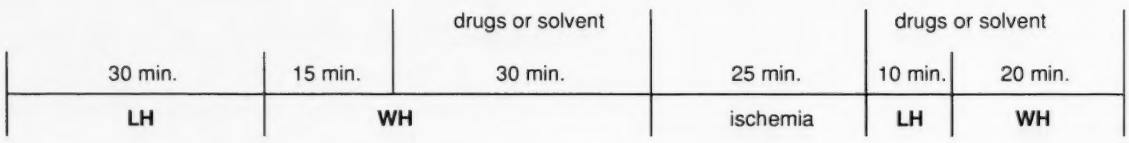


Fig. 1. Experimental protocol.

2.4. Statistical analysis

Differences between solvent and drug treated hearts were assessed with the Mann-Whitney U-test. Two tailed probabilities of less than or equal to 0.05 were considered as being significant. To evaluate the effects of treatment on baseline variables, differences with values prior to drug administration were subjected to statistical analysis.

The effect of the different treatments on the ischemic response was evaluated by using the absolute values obtained after the insult.

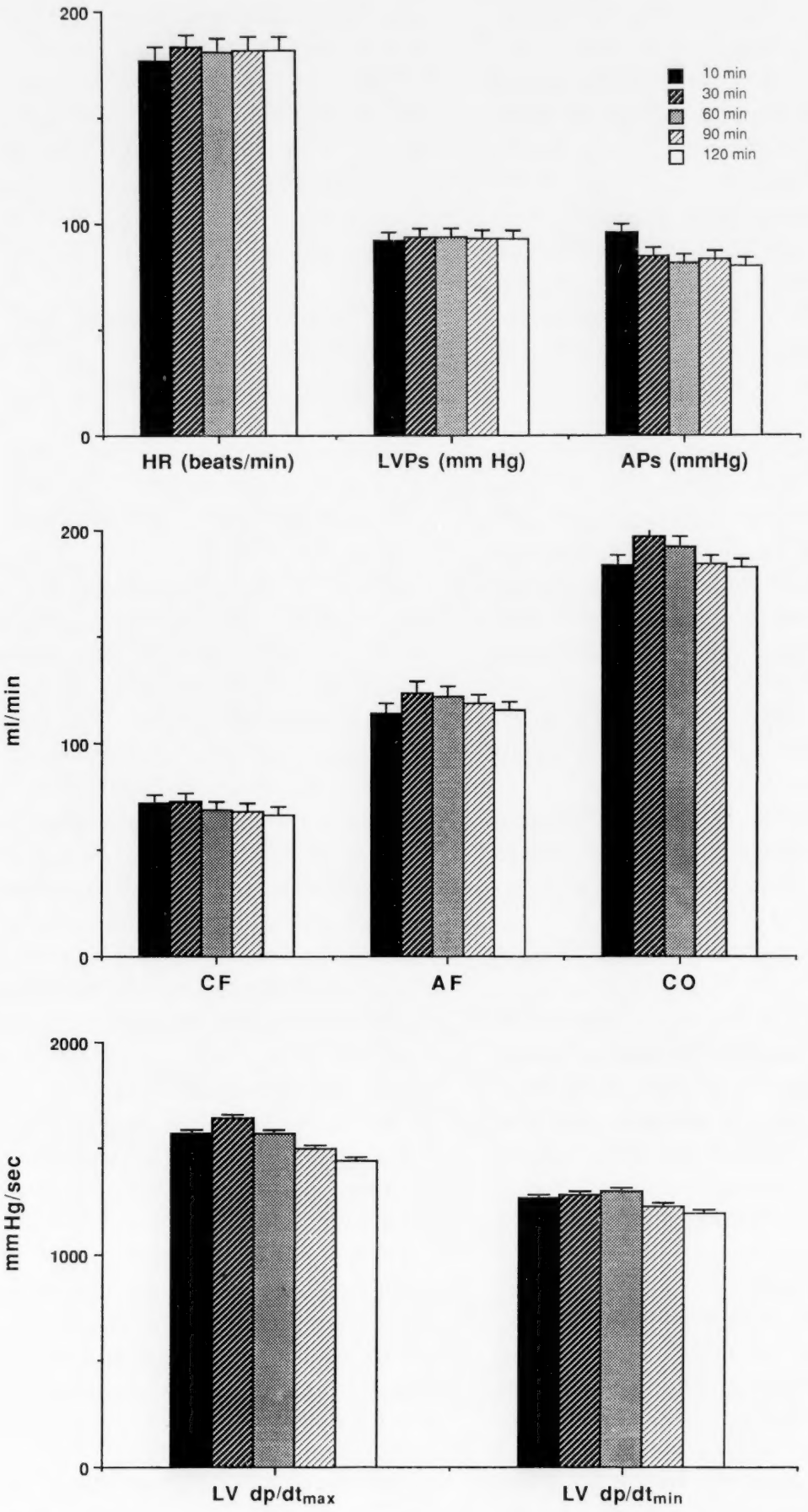


Fig. 3. Evolution of the cardiodynamic parameters during 2 h working heart condition. HR: heart rate, LVP: left ventricular pressure, AP: aortic pressure, AF: aortic flow, CF: coronary flow, CO: cardiac output, LVdp/dt: first derivative of the left ventricular pressure. LH: Langendorff heart; WH: working heart.

3. Results

3.1. Hemodynamic study

3.1.1. Control experiment

Control experiments without ischemia (n = 7) were performed to test the stability of the system. The hemodynamic stability of the preparation was checked for 2 h in the working state, and cardiodynamic parameters, recorded every 10 min, were stable during the entire observation period (fig. 3). Values at the end of the experiment were not significantly different from the initial starting values ($P > 0.05$).

3.1.2. Starting values in the experimental groups

Comparison of the pre-ischemic values for the different cardiodynamic parameters were made. No statistically significant differences in starting values of the cardiodynamic parameters were found between the four experimental groups.

3.1.3. Pre-ischemic effects of solvent and drugs on cardiodynamic parameters

Administration of solvent prior to ischemia did not induce significant changes in cardiodynamic function (fig. 4). As can be seen, R 56 865 $4 \cdot 10^{-7}$ M and verapamil $1.5 \cdot 10^{-8}$ M induced no effects on HR and LVP, AP or flow values. LV dp/dt_{max} was decreased to 92 and 82% of the control values in the R 56 865 ($P > 0.05$) and verapamil $1.5 \cdot 10^{-8}$ M groups ($P < 0.05$) respectively. Verapamil at 0.016 mg/l induced a significant depression of LVdp/dt, AF and CO values ($P < 0.005$). Although at this dose there was also a slight depression of the other parameters, the differences from control values were not statistically significant.

3.1.4. Post-ischemic cardiovascular recovery: effects of solvent and drugs

As a first step the period of ischemia needed to obtain minimal recovery in solvent experiments was evaluated. Twenty minutes appeared too short since about 80% recovery of most parameters was obtained (fig. 5, upper). After 30 min of ischemia followed by 30 min of reperfusion nearly all hearts went into hypercontracture ('stone heart') and failed to perform any function at all (fig. 5, lower). Thus 25 min of global normothermic ischemia appeared an acceptable ischemic period, after which the mean recovery of cardiodynamic function was severely depressed: one out of seven hearts developed hypercontracture and one fibrillated upon reperfusion; two out of seven did not show any recovery of contractile function at all and the remaining three showed a minimal degree of recovery. Only one heart developed enough pressure to result in aortic flow.

The influence of drugs or solvent on post-ischemic recovery of cardiodynamic function is shown in fig. 6. After 25 min of global ischemia followed by 30 min of reperfusion, a marked drop in HR, LVP, AP, CF, AF, CO, $LVdp/dt_{min}$ and $LVdp/dt_{max}$ was observed in the two verapamil groups but not in the R 56 865 group. With verapamil at $1.5 \cdot 10^{-8}$ M, some degree of protection was obtained but obviously less than in the R 56 865 group. In the R 56 865 group ($4 \cdot 10^{-7}$ M) the mean recovery was more than 80% for HR, LVP, and $LVdp/dt_{min}$ and more than 70% for AP and CF. $LVdp/dt_{max}$ values returned to 67% and mean values of 33 and 48% were obtained for AF and CO, respectively. Mann-Whitney U-tests showed that functional recovery after ischemia was highly improved after pretreatment with R 56 865 as compared to solvent and both verapamil groups ($P < 0.005$).

3.2. Morphological changes and calcium shifts after postischemic reperfusion

3.2.1. Control experiments

Hearts perfused with oxygenated KH for 2 h had a normal ultrastructure. The subcellular structures were well preserved, apart from the occurrence of some extracellular fluid, probably caused by perfusion with a solution devoid of proteins. The glycocalyx was closely apposed to the sarcolemma, the latter surrounding the cell without discontinuities. Mitochondria were regularly arranged between the sarcomeres and contained many osmiophilic granules, which are typical for well-oxygenated cells (fig. 7). The nucleus contained evenly distributed chromatin. Glycogen granules were abundantly present in the cytosol. Calcium deposits, visible as 20 nm particles, were confined to the inner leaflet of the sarcolemma (fig. 7), the T-tubules and intercalated disks. Single deposits were sometimes observed in the mitochondria, whereas all other organelles were devoid of calcium precipitate. The subcellular distribution of calcium deposits was similar between the different (endo-, mid- and epicardial) areas.

3.2.2. Solvent experiments

In solvent-treated hearts, the functional deterioration observed upon reperfusion after 25 min of ischemia was accompanied by ultrastructural changes and shifts in cytochemically detectable calcium. Changes were observed in the whole ventricular wall, except in the outermost epicardium; they varied from mild, i.e. loss of the mitochondrial matrix granules, swelling of the mitochondrial matrix, intracellular edema, accumulation of single calcium deposits in the mitochondria, to more pronounced alterations involving other cell organelles.

In severely damaged cells, sarcomeres were mostly hypercontracted (fig. 8a) and nuclei were pyknotic, with margination of the heterochromatin. In these cells, the sarcolemma and the T-tubules lost their calcium

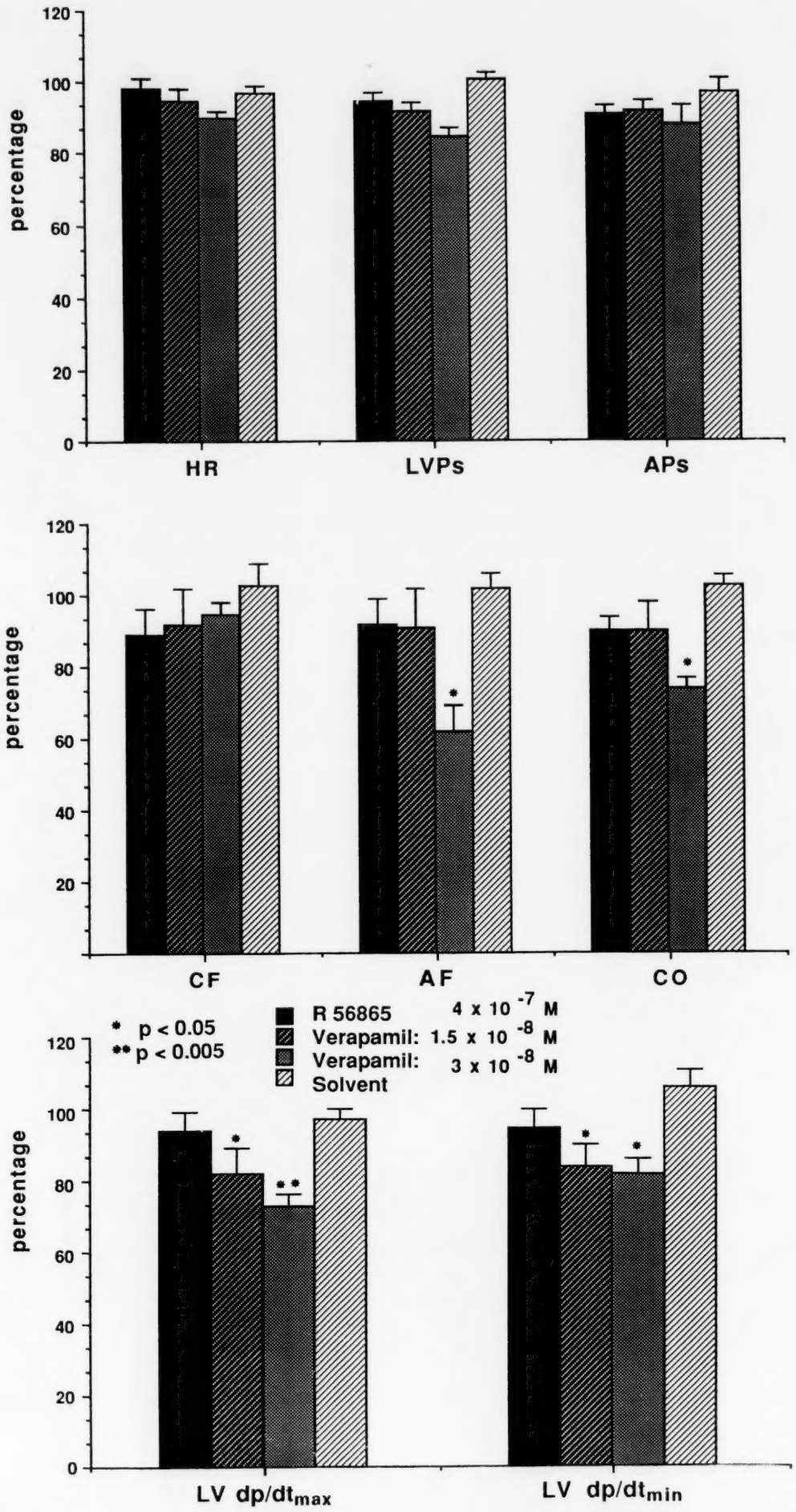
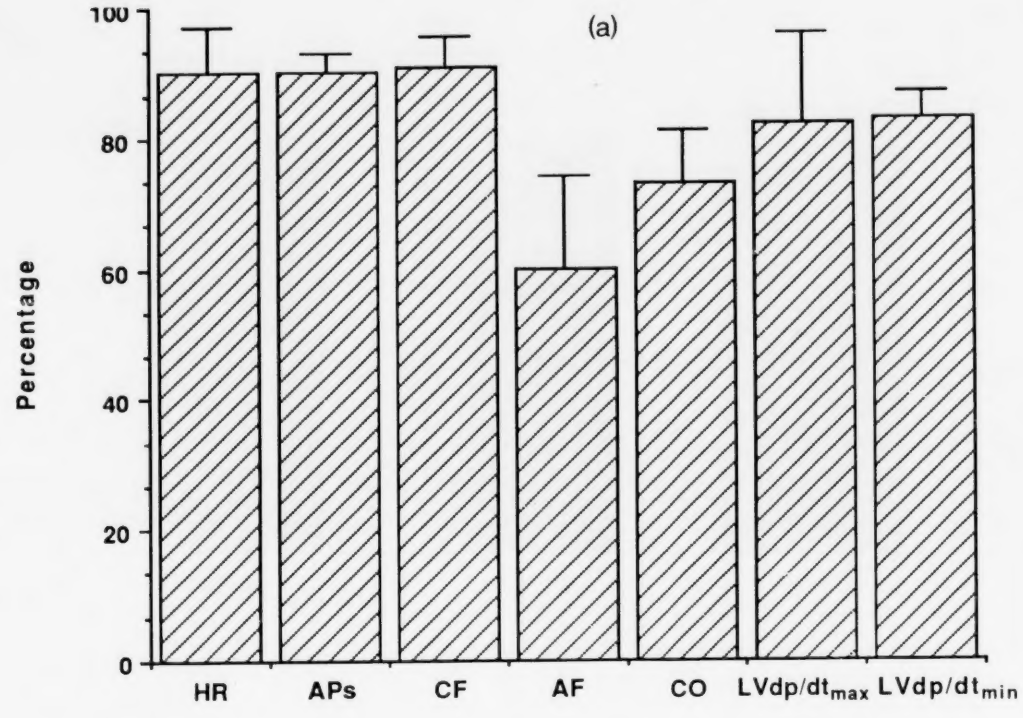


Fig. 4. Pre-ischemic effect of solvent and drugs on cardiodynamic function. HR: heart rate, LVP: left ventricular pressure, AP: aortic pressure, AF: aortic flow, CF: coronary flow, CO: cardiac output, LVdp/dt: first derivative of the left ventricular pressure.

binding capacity whereas the mitochondria accumulated large amounts of calcium precipitate, either as single dots or as large clusters (fig. 8).

The most damaged areas showed massive degeneration of the cells, as characterized by contraction band necrosis, pyknosis of nuclei, discontinuities in the sar-



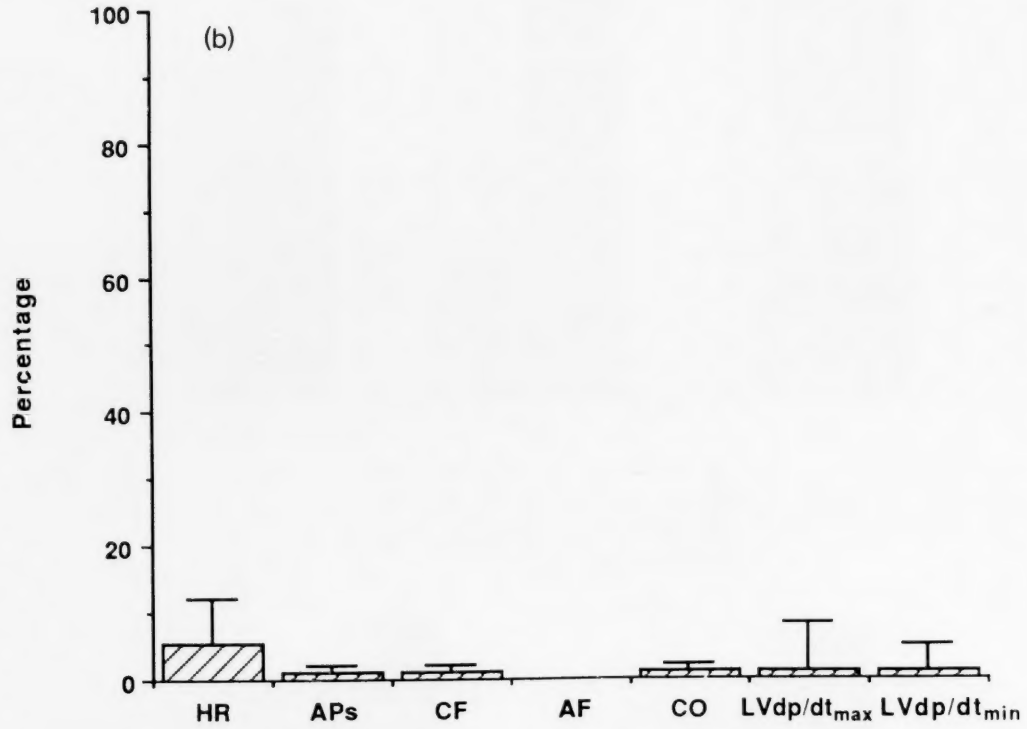


Fig. 5. Recovery of cardiodynamic parameters after 20 min (a) and 30 min (b) of ischemia followed by 30 min of reperfusion. HR: heart rate, LVP: left ventricular pressure, AP: aortic pressure, AF: aortic flow, CF: coronary flow, CO: cardiac output, LVdp/dt: first derivative of the left ventricular pressure.

colemma and mitochondria containing flocculent densities (Jennings granules). Mitochondria altered in this way usually did not contain calcium precipitate.

3.2.3. Effect of pretreatment with R 56 865 and verapamil

In the verapamil-pretreated groups, changes qualitatively and quantitatively similar to those described in the solvent-treated group were present (fig. 9a). In the R 56 865-treated group, signs of irreversible damage (nuclear pyknosis, flocculent densities in mitochondria, contraction band necrosis) were restricted to a limited number of cells in the intermediate zone. Some cells had an ultrastructure approaching that of pre-ischemic cells. In other cells, the mitochondria sometimes showed disorganization of the cristae and a decreased number of osmiophilic granules in contrast to solvent-treated hearts.

Verapamil-pretreated hearts with severe ultrastructural damage also showed calcium shifts similar to those in solvent-treated hearts, i.e. loss of sarcolemma-bound calcium deposits with either simultaneous clustered deposits in mitochondria or without mitochondrial calcium accumulation. In the latter, the mitochondria were characterized by the presence of flocculent densities and the sarcolemma often showed

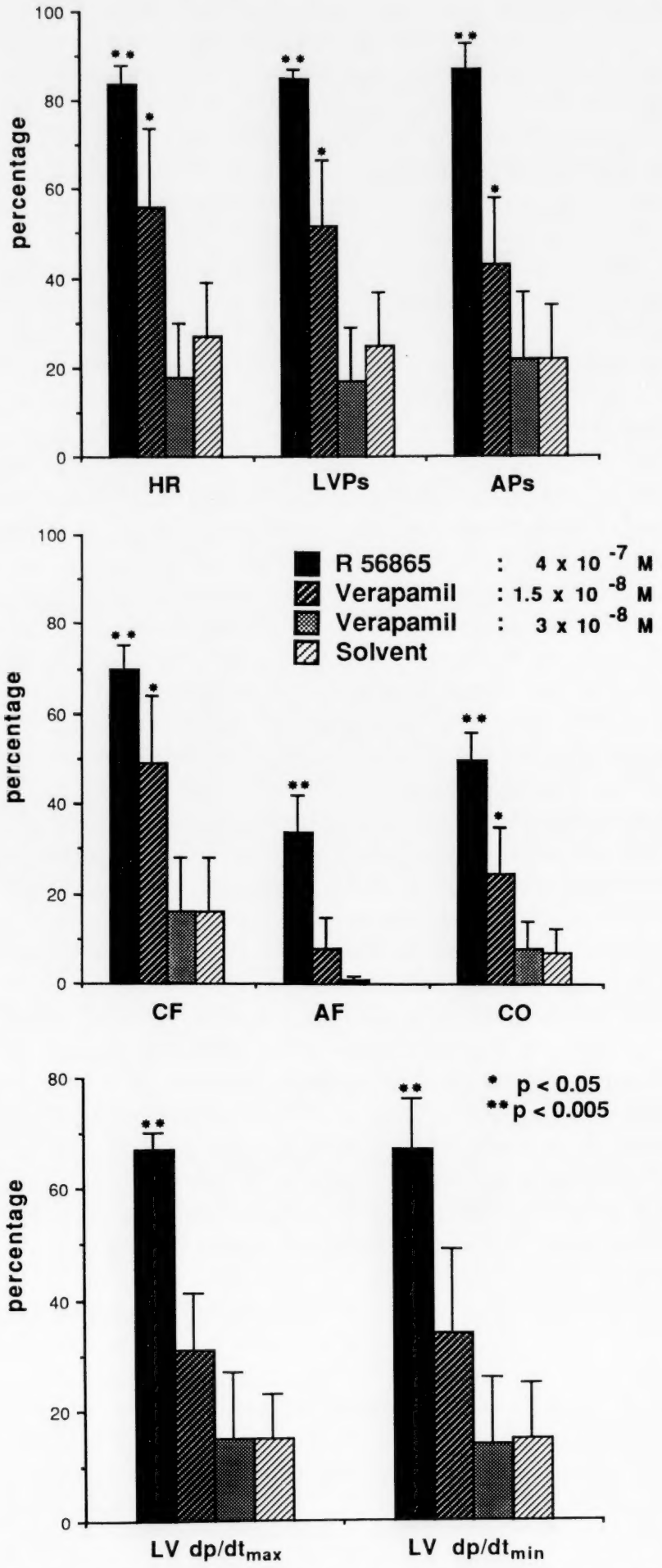


Fig. 6. Effects of R 56 865 or verapamil on recovery of cardiodynamic parameters after 25 min of ischemia followed by 30 min of reperfusion. HR: heart rate, LVP: left ventricular pressure, AP: aortic pressure, AF: aortic flow, CF: coronary flow, CO: cardiac output, LVdp/dt: first derivative of the left ventricular pressure.

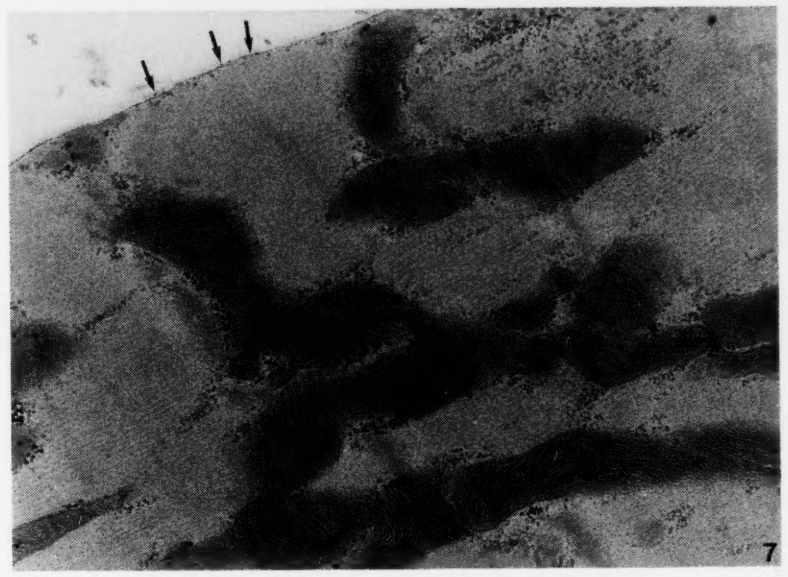


Fig. 7. Localization of calcium in subendocardial muscle after 120 min of normoxic perfusion. Calcium deposits along the sarcolemma and T-tubules (t) appear to be associated with the sarcolemma (arrow). Mitochondria (m) contain regularly arranged crystae and many osmiophilic granules (open arrowheads) (×39150); e: endothelium.

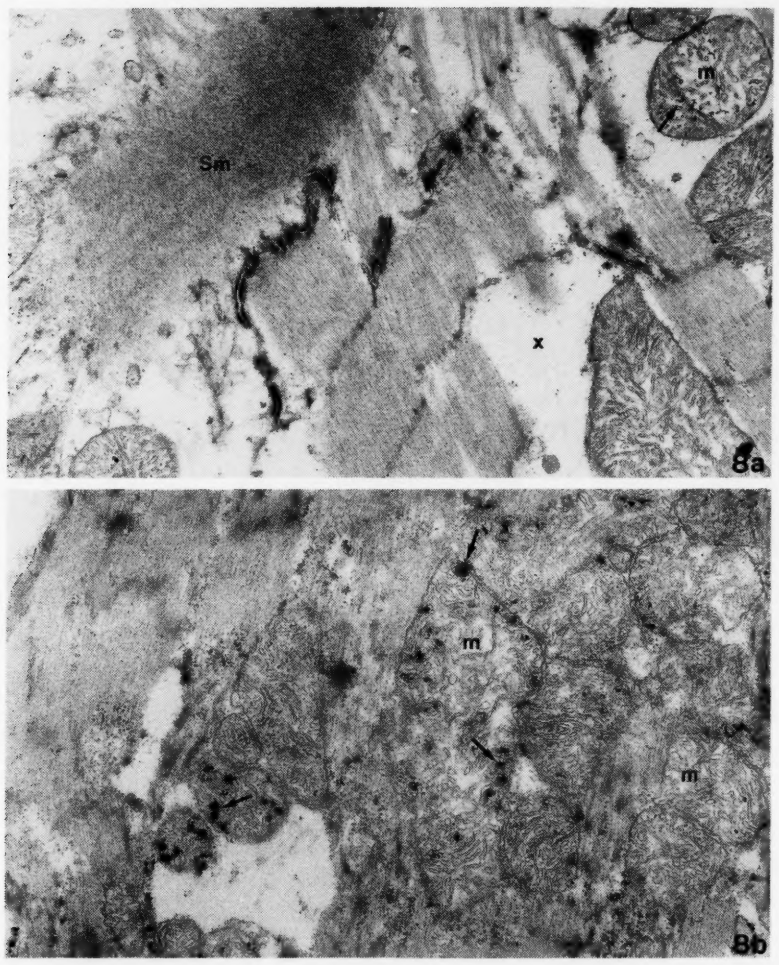


Fig. 8. Calcium localization after 25 min of ischemia followed by 30 min of reperfusion. (a) Sarcomeres (Sm) are contracted and intracellular edema is (×) present. Mitochondria (m) are swollen and contain single calcium deposits (arrow) (×22020). (b) A severely damaged cell shows swollen mitochondria (m) containing clusters of calcium deposits (arrows). The sarcolemma (Sl) is devoid of precipitate. (×26580), e: endothelium.

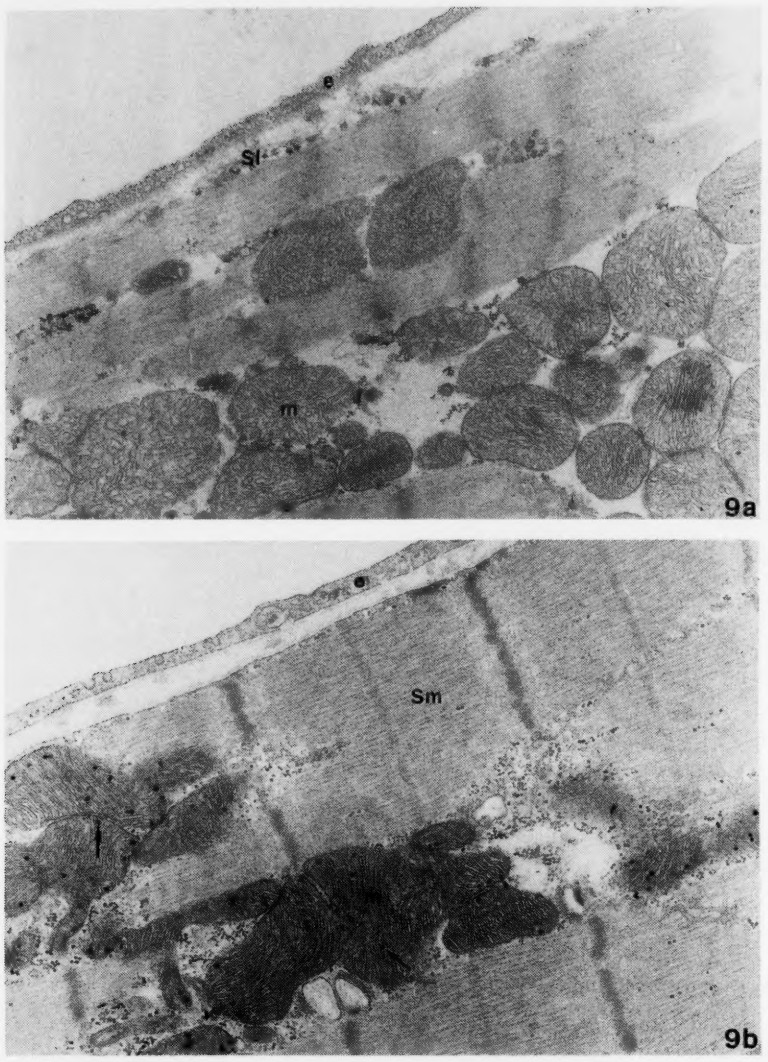


Fig. 9. Cardiac muscle after 25 min of ischemia followed by 30 min reperfusion. (a) Pretreatment with verapamil ($3 \cdot 10^{-8}$ M). As in solvent-treated tissue, mitochondria (m) are swollen and contain calcium precipitate (arrow). The sarcolemma (Sl) is devoid of precipitate (×21290). (b) Pretreatment with R 56 865 ($4 \cdot 10^{-7}$ M). As in control tissue, mitochondria (m) contain regularly arranged cristae and many osmiophilic granules (arrow). The number of sarcolemma-bound calcium deposits is reduced when compared to control tissue. (×32400), e: endothelium, Sm: sarcomeres.

discontinuities (fig. 9a).

Structural protection with R 56 865 was paralleled by an almost normal pattern of calcium distribution. Most cells of the R 56 865 pretreated hearts showed calcium deposits associated with the sarcolemma, T-tubules and intercalated disks. However, in some of the cells the number of sarcolemma-bound deposits was less than in cells of the control group (fig. 9b).

4. Discussion

In this study, global normothermic ischemia in the isolated working rabbit heart was used to explore the role of calcium in the pathogenesis of ischemic cell death. For this purpose, direct as well as indirect methods were applied and the criteria used for the evaluation of protection were cardiodynamic function and ultrastructure. All measured cardiodynamic values in the working rabbit heart remained almost constant during the experiment, indicating that the system was accurate and reproducible. In this experimental set-up, an afterload (50 mm Hg) and the ability of the heart to overcome this afterload resulted in aortic flow. Therefore, the amount of aortic flow, which is a reflection of the performance of the heart, was the most sensitive parameter of functional recovery. When the hearts were subjected to 25 min of ischemia, minimal recovery of functional cardiodynamic parameters was observed. This time period was therefore chosen to evaluate the effects of cardioprotective drugs.

As a direct approach to evaluate the role of calcium in ischemic cell death we used a cytochemical procedure. Indeed, biochemical assays measure total tissue or total cell calcium and there is no correlation between these parameters and the free intracellular calcium concentration (Cheung et al., 1986; Murphy et al., 1983). Furthermore, transmural biopsies from ischemic areas contain a mixture of viable and infarcted cells and averaged results from such transmural biopsies need careful interpretation. The cytochemical approach used in this study makes it possible to localize calcium in specific areas i.e. infarcted, viable ischemic and non-ischemic areas. Using this PPA technique, calcium has been localized (20 nm particles) at the inner leaflet of the sarcolemma T-tubules and intercalated disks of the myocardial cells in man and several animal species (Borgers and Piper, 1986; Borgers et al., 1987; Olbrich et al., 1988). These calcium deposits are thought to represent the calcium pool bound to acidic phospholipids. The existence of a sarcolemmal calcium pool and its participation in calcium loading during inotropic interventions has been documented (Wheeler Clark and Torney, 1987).

It has been proposed that the calcium bound to the inner side of the lipid bilayer plays an important role in the integrity of the sarcolemma (Borgers et al., 1987). The loss of the ability of the sarcolemma to bind calcium is considered a first sign of loss of cellular calcium homeostasis because it already occurs during the ischemic phase (prior to reperfusion) and precedes other signs of membrane degeneration (Borgers et al., 1987).

To examine possible pathways of calcium entry under these experimental conditions, we compared the effects of verapamil, a slow channel blocker, with those of R 56 865, which has little effect on calcium channels (Carmeliet et al., 1991; Himmel et al., 1990; Koch et al., 1990). During the pre-ischemic period, R 56 865 at the concentration of $4 \cdot 10^{-7}$ M exerted no significant effect on the cardiodynamic parameters measured, such as HR and contractility. This rules out the possibility of an energy sparing effect in the pre-ischemic period as a mechanism of cardioprotection upon postischemic reperfusion. Verapamil significantly reduced contractility ($LVdp/dt_{max}$) in a dose dependent manner, an

effect which is consistent with the L-type Ca^{2+}-channel inhibiting property of this drug.

Functional data indicated that R 56 865, but not verapamil, provides protection against ischemic damage. These findings are in agreement with previous studies where R 56 865 was shown to protect against ouabain-induced cardiac toxicity in various species (Vollmer et al., 1987; Schneider et al., 1987, 1988; Finet et al., 1989) and against functional defects elicited by ischemia or hypoxia (Garner et al., 1989, 1990; Borgers and Ver Donck, 1990; Koch et al., 1990). Recently, the compound has also been shown to suppress reperfusion-induced arrhythmias potently (Schleuffer et al., 1990) as well as triggered arrhythmias and propagated contractions (Daniels and ter Keurs, 1990). It is not clear if the protective effects observed during ischemia can be explained by a mechanism similar to that by which the drug exerts protective effects against ouabain toxicity.

In isolated cardiomyocytes from rat and rabbit, R 56 865 prevents contractions elicited by pathological stimuli, such as veratridine, singlet oxygen, lysophosphatidylcholine, ouabain (Borgers and Ver Donck, 1990). These stimuli induce irreversible hypercontracture as a consequence of intracellular calcium overload. This hypercontracture can be prevented by tetrodotoxin (inhibitor of fast sodium channel) and by reduced extracellular sodium, but not by nifedipine. Thus the calcium overload induced by these stimuli could be explained by a dysfunction of the fast sodium channel, resulting in sodium and, subsequently, calcium overload by sodium/calcium exchange (Borgers and Ver Donck, 1990). In conclusion, the cytochemical data indicate that the subcellular calcium distribution changes during myocardial ischemia. Furthermore, the protective effects observed with R 56 865 but not with verapamil indicate that the bulk of calcium responsible for the calcium overload is not entering the cell through slow channels.

Acknowledgements

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References

Borgers, M. and L. Ver Donck, 1990, R 56 865, a new cardioprotective principle based on prevention of ion channel pathology, J. Mol. Cell. Cardiol. 22 (Suppl. 1), 4.

Borgers, M., G.S. Liu, R. Xhonneux, F. Thoné and P. Van Overloop, 1987, Changes in ultrastructure and Ca^{2+} distribution in the isolated working heart after ischemia, Am. J. Pathol. 126, 92.

Borgers, M. and H.M. Piper, 1986, Calcium shifts in anoxic cardiac myocytes: a cytochemical study, J. Mol. Cell. Cardiol. 18, 439.

Borgers, M., F. Thoné and L. Ver Donck, 1985, Sarcolemma-bound calcium. Its importance for cell viability, Basic Res. Cardiol. 80 (Suppl.1), 31.

Borgers, M., F. Thoné, A. Verheyen and H.E.D.J. Ter Keurs, 1984, Localization of calcium in skeletal and cardiac muscle, Histochem. J. 16, 295.

Boskey, A.L. and A.S. Posner, 1982, Optimal conditions for Ca-acidic phospholipid-PO_4 formation, Calcif. Tissue Int. 34, S1.

Carmeliet, E. and J. Tytgat, 1991, Agonistic and antagonistic effects of R 56 865 on the Na^+ channel in cardiac cells, Eur. J. Pharmacol. 196, 53.

Cheung, J.V., J. Bonventre, C.D. Malis and A. Leaf, 1986, Calcium and ischemic injury, N. Engl. J. Med. 314, 1670.

Chien, K.R., J. Abrams, A. Semoni, J.T. Martin and J.L. Faber, 1978, Accelerated phospholipid degradation and associated membrane dysfunction is irreversible ischemic liver cell injury, J. Biol. Chem. 253, 4809.

Daniels, M.C.G. and H.E.D.J. Ter Keurs, 1990, Triggered propagated contractions and triggered arrhythmias in rat cardiac trabeculae are suppressed by R 56 865 (abstract), Circulation 81 (Suppl. III), 143.

Finet, M., G. Bravo and T. Godfraind, 1989, The protective action of R 56 865 against ouabain induced intoxication in rat heart isolated atria and ventricles, Eur. J. Pharmacol. 164, 555,

Garner, J.A., M. Bernier and D.J. Hearse, 1989, R 56 865 abolishes reperfusion-induced arrhythmias in vivo without altering hemodynamics, Circulation II, 501.

Garner, J.A., M. Bernier and D.J. Hearse, 1990, R 56 865, a potent new anti-arrhythmic agent, effective during ischemia and reperfusion in the rat heart, J. Cardiovasc. Pharmacol. 16, 468.

Himmel, H.M., D. Wilhelm and U. Ravens, 1990, Effects of R 56 865 on membrane currents in isolated ventricular myocytes in the guinea pig, Eur. J. Pharmacol. 187, 235.

Katz, A.M. and H. Reuter, 1979, Cellular calcium and cardiac cell death, Am. J. Cardiol. 44, 188.

Koch, P., B. Willfert and Th. Peters, 1990, R 56 865, a new anti-ischemic principle, Cardiovasc. Drug Rev. 8, 238.

Langendorff, O., 1895, Untersuchungen am uberlebenden Saugetierherzen, Pflügers Arch. 61, 291.

Lu, H.R., G. Vandeplassche, L. Wouters and M. Borgers, 1989, Beta blockade in the ischemic reperfused working rabbit heart dissociation of beta-adrenergic blocking and protective effects, Arch. Int. Pharmacodyn. Ther. 301, 165.

Murphy, E., J.F. Acton, C.R. Horres and M. Lieberman, 1983, Calcium elevation in cultured heart cells its role incell injury, Am. J. Physiol. 245, C316.

Nayler, W.G., S. Papagiotopaulos, J.B. Elz and M.J. Daly, 1988, Calcium mediated damage during post-ischemic reperfusion, J. Mol. Cell. Cardiol. (Suppl. II), 41.

Olbrich, H.G., M. Borgers, F. Thoné, M. Frotscher, E. Mutschler, M. Schneider, G. Kober and Kaltenbach, 1988, Ultrastructural localization of calcium in the myocardium of cardiomyopathic syrian hamsters, J. Mol. Cell. Cardiol. 20, 753.

Poole-Wilson, P.A., D.P. Harding, P.D.V. Bourdillon and M.A. Tones, 1984, Calcium out of control, J. Mol. Cell. Cardiol. 16, 175.

Schleuffer, E., A. Mozes, I. Guttmann and T. Peters, 1990, R 56 865 processes anti-ischemic properties even when given only during reperfusion, in: Ionic Currents and Ischemia, eds. J. Vereecke, P.P. Van Bogaert and F. Verdonck (Leuven University Press, Leuven) p. 301.

Schneider, J., E. Beck and E. Scheufer, 1987, Protective effects of R 56 865 on cardiac glycoside intoxication, J. Mol. Cell. Cardiol. 19 (Suppl. III), S 84.

Schneider, J., E. Beck and M. Borgers, 1988, Dose dependent

protection R 56 865 against digitalis toxicity in the guinea pig heart lung preparation, Naunyn-Schmiedeb. Arch. Pharmacol. 337, R 65.

Shen, A.C. and R.B. Jennings, 1972, Myocardial calcium and magnesium in acute ischemic injury, Am. J. Pathol. 67, 417.

Vollmer, B., C.H. Meuter, R. Stockbroeckx and P.A.J. Janssen, 1987, R 56 865 prevents electrical and mechanical signs of ouabain intoxication in guinea pig pappilary muscle, Eur. J. Pharmacol. 142, 137.

Wheeler-Clark, E.S. and J.McD. Torney, 1987, Electronprobe X-ray microanalysis of sarcolemmal and functional sarcoplasmic reticulum in rabbit papillary muscles low sodium induced calcium alterations, Circ. Res. 60, 247.

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Short communication

Neurosteroids block Ca^{2+} channel current in freshly isolated hippocampal CA1 neurons

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Certain synthetic and endogenous steroids are known to modulate neuronal responses to γ-aminobutyric acid (GABA) and also change the firing frequency of certain neurons. However, there is nothing known of the effect(s) of steroids on voltage-gated calcium currents in mammalian neurons. We show here that the steroids (0.1-100 μM) allotetrahydrocorticosterone (THCC), dehydroepiandrosterone sulfate (DHEAS) and pregnanolone can rapidly and reversibly depress voltage-gated calcium currents in freshly isolated adult hippocampal CA1 pyramidal neurons. This blocking action occurs in the presence of picrotoxin (10 μM). Tail current analysis shows that THCC appears to be a ligand that selectively and reversibly depresses the ω-conotoxin (fraction GVIA) sensitive portion of the calcium current. These results demonstrate that certain steroid metabolites have a direct membrane site of action which may influence brain excitability.

Steroids; Ca^{2+} currents; Hippocampus CA1 neurons; Picrotoxin; Patch clamp (whole cell)

1. Introduction

The presence of steroids in the mammalian central nervous system (CNS) is well documented and the synthesis of some steroids, neurosteroids, and their sulfate derivatives occurs primarily in glial cells (Baulieu and Robel, 1990). Steroids exert both genomic and non-genomic effects on cells, the latter reported as being rapid with a direct action on neural membranes which is independent of the effects of nuclear transcription (McEwan, 1991). The ability of some steroids to influence brain activity is well known, particularly as certain endogenous and synthetic steroids are potent and specific modulators of the $GABA_A$ receptor. Extra- and intracellular recordings, both in vitro and in vivo, have demonstrated that steroids can rapidly alter the firing rate of neurons (for review, see McEwan, 1991). In addition, steroids have been reported to affect neurosecretion, such as either increasing or decreasing dopamine release (see McEwan, 1991). In the peripheral nervous system, the steroid anesthetic alphaxalone rapidly and reversibly blocked sodium and potassium currents (Benoit et al., 1988). The 3α-hydroxy ring A-reduced metabolites of progesterone and deoxycorticosterone, such as 5β-pregnan-3α-OL-20-one (pregnanolone) and 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydrodeoxycorticosterone; THCC) have been shown to potentiate γ-aminobutyric acid ($GABA_A$)-gated Cl^- current (Majewska et al; 1986). In contrast, dehydroepiandrosterone sulfate (DHEAS) reversibly antagonizes the $GABA_A$-gated Cl^- current, acting as an allosteric antagonist of the $GABA_A$ receptor (Majewska et al., 1990). Considering that steroid hormones are known to influence brain excitability, we therefore examined whether steroids known to modulate $GABA_A$ receptors as well as the related compound, THCC, could modulate voltage-gated calcium channels in isolated hippocampal neurons.

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2. Materials and methods

Pyramidal neurons were acutely isolated from the CA1 region of the mature guinea-pig hippocampus according to the methods described by Kay and Wong (1986). Whole-cell patch-clamp Ca^{2+} channel currents were recorded at room temperature (20–25 °C) using Ba^{2+} as the external charge-carrying cation. The bath solution contained (in mM; except where noted): $BaCl_2$ 3; tetraethylammonium chloride (TEA-Cl) 140; $MgCl_2$ 1; HEPES 10; glucose 6; tetrodotoxin (TTX) 2 μM; pH of 7.4 and an osmolality of 320 mOsm/kg H_2O. The patch pipette solution contained (in mM): N-methyl-

D-glucamine chloride 120; Cs_4-BAPTA 5; Mg-ATP 5; Tris-phosphocreatine 20; creatine kinase (20 U/ml); pH 7.2 and an osmolality of 315 mOsm/kg H_2O. Pipette-to-bath resistances prior to seal formation were typically 3–7 MΩ; seal resistances were >10 GΩ. Following seal formation and prior to entering the whole-cell mode, electrode capacitance was neutralized by using the capacitance compensation circuitry of the Axopatch-1D amplifier (Axon Instruments). In the whole-cell mode, the amplifier was further adjusted to correct for 80–85% of the series resistance (2–4 MΩ). Acquisition and analysis was with pClamp 5.51 software (Axon Instruments). Currents were evoked from a holding potential (V_H) of -80 mV with either 10 and/or 200 ms depolarizing steps to a test potential (V_T) of -10 mV at 10 and 30 s intervals respectively, filtered at 10 kHz (-3 dB, 8-pole low pass Bessel filter) and digitally sampled at 500 μs per point and 50 μs per point for tail currents. Capacitative and leakage currents were digitally subtracted out on-line from all records; measurement was on the average of three traces. Tail current amplitudes were estimated by fitting the falling phase of the current to a single exponential function and extrapolating the curve to zero time using NFIT (Island Products, TX). Stock solutions of the steroids were prepared in ethanol; final ethanol volume never exceeded 0.01%. A rapid superfusion system was used where a six barreled array of individual tubes (each tip diameter 200 μM) was positioned near the cell under study with drug solutions applied through the pipette by gravity feed; flow was controlled with a solenoid valve. The recording chamber was continuously perfused at all times for all experiments with the normal (drug free) solution at a flow rate of 1.5 ml/min. For the quantitation of block, peak current values were used. Percent block was determined according to the formula $100 \times (1 - I_{drug}/I_{control})$, where $I_{control}$ is the leak subtracted peak current amplitude prior to the drug application and I_{drug} is the leak subtracted peak current amplitude in the presence of the test drug. The I_{drug} value used in the calculation of percent block was obtained after being at a steady state value for 1 min. All quantitative data are expressed as means $\pm$ S.E.M. All chemicals were purchased from Sigma (St. Louis, MO) except ω-conotoxin (CgTX) from Peninsula Laboratories (Belmont, CA).

3. Results

Depolarizing voltage steps from V_H -80 mV to a test potential (V_T) of -10 mV, typically evoked a partially inactivating Ca^{2+} current, as illustrated in fig. 1A (control). Whole-cell patch clamp recordings have suggested that this high-voltage activated Ca^{2+} current is comprised of an inactivating N- and a non-inactivating L-type calcium channel (Doerner et al., 1988). For the cell illustrated in fig. 1A, THCC depressed the inward Ca^{2+} current in a concentration-dependent manner, with a 14, 47 and 54% block of the peak current and the late portion of the current (measured 10 ms before the end of the voltage step) was depressed 10, 37 and 50% at 0.1, 1.0 and 10 μM respectively. The current rapidly reversed to 91% of control following perfusion with control solution after applica-

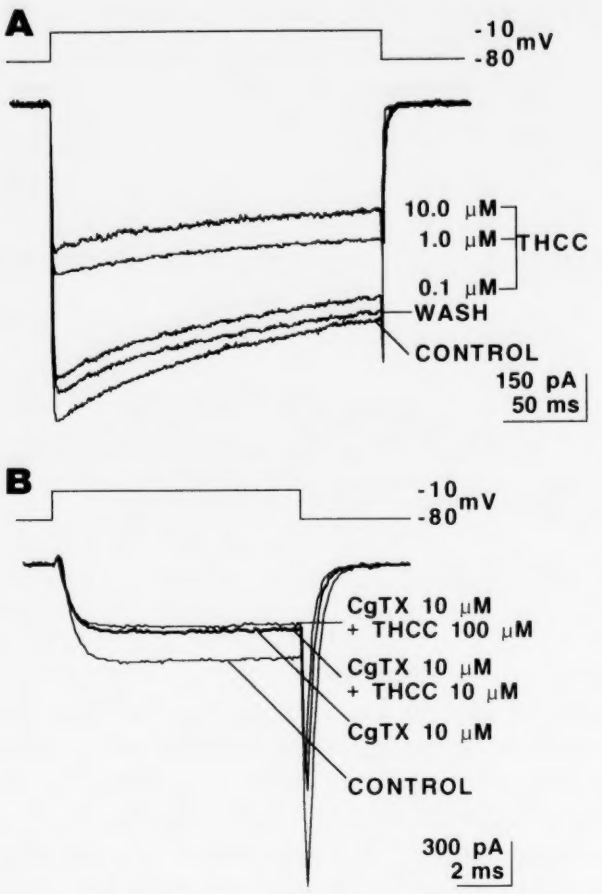

Fig. 1. Effect of the steroid allotetrahydrocorticosterone (THCC) on the voltage-gated Ca^{2+} current in a hippocampal CA1 neuron. (A) Concentration-dependent block of the Ca^{2+} current by THCC at -80 mV, evoked by a 200 ms depolarizing step to -10 mV, followed by wash with drug free solution. (B) Peak and tail currents at -80 mV following a 10 ms depolarizing step to -10 mV. THCC CgTx block the same fraction of Ca^{2+} current. In the presence of a saturating CgTx concentration (10 μM) for maximal block, 10 μM THCC had no effect; at 100 μM, THCC gave a small additional block on the peak but not the tail current. (A) and (B) different cells; see text for details.

TABLE 1

Inhibition of the voltage-gated Ca^{2+} channel current.

Values are means $\pm$ S.E.M.; ( ), number of cells tested; $-$, not tested; V_{HOLD}, -80 mV; V_{TEST}, -10 mV.

	% block peak current				
	0.01 μM	0.1 μM	1.0 μM	10.0 μM	100.0 μM
THCC	7.2 $\pm$ 1.6 (4)	16.7 $\pm$ 2.1 (4)	32.5 $\pm$ 2.2 (5)	50.1 $\pm$ 2.7 (5)	58.5 $\pm$ 2.4 (4)
DHEAS	–	10.2 $\pm$ 1.9 (4)	20.7 $\pm$ 2.1 (4)	43.1 $\pm$ 3.8 (4)	73.3 $\pm$ 5.6 (4)
Pregnanolone	–	13.5 $\pm$ 2.5 (4)	20.1 $\pm$ 7.0 (4)	40.3 $\pm$ 4.4 (5)	77.8 $\pm$ 2.5 (5)

tion of 10 μM THCC (fig. 1A). The blocking action of THCC was rapid, acquiring steady state values at each concentration within 60–120 s. We also examined the action of DHEAS and pregnanolone; the maximal depressant action on peak current is summarized in table 1. For all the neurosteroids examined, the Ca^{2+} current was reversibly blocked, returning with wash to 85–100% of control through 10 μM; >10 μM gave a 60–80% recovery on some, but not all cells following perfusion with control solution. Alphaxalone was weakly effective with only a 29% block at 100 μM ($n = 3$). Perfusion with 10 μM picrotoxin (PTX) had no effect on the Ca^{2+} current and did not prevent the blocking action of THCC, thus, eliminating the possibility of $GABA_A$ receptor involvement ($n = 3$). Analysis of the peak current depression by THCC (0.01, 0.1 and 1.0 μM) in the presence of PTX demonstrated a similar amount of block as to THCC alone, 5.3 ± 0.9, 18.3 ± 1.8 and 40 ± 3.7% respectively (compare with table 1).

Recent experiments have suggested that CgTX blocks the inactivating N-type and not the non-inactivating L-type Ca^{2+} current (Plummer et al., 1989). It has been shown that CgTX blocks a fraction, both the peak and late, of the Ca^{2+} current that is N-like but not the L- or the resistant (R)-type in hippocampal CA3 (Mogul and Fox, 1991) and CA1 neurons (Regan et al., 1991). In our hands, 10 μM CgTX blocked a fraction of the Ca^{2+} current to give a maximal and irreversible block of 35 ± 2% of the peak and 32 ± 5% of the late portion of the Ca^{2+} current ($n = 7$). Tail currents have been successfully used to pharmacologically isolate the various components of the whole-cell Ca^{2+} current (Regan et al., 1991). This is illustrated in fig. 1B, where the current elicited by a 10 ms voltage step from -80 to -10 mV and measured at -80 mV, shows peak and tail currents. In this neuron CgTX irreversibly blocked the peak current 29%. In the presence of 10 μM CgTX, application of 10 μM THCC showed no effect ($n = 4$). A small additional blocking effect (4%) was seen with subsequent addition of 100 μM THCC ($n = 2$). Tail currents at -80 mV were well fit by a single exponential function (control; $\tau = 260$ μs). There was a comparable block of the peak tail current (21%) with the peak current (29%). The partialy blocked tail current in the presence of CgTX was not further affected by THCC ($\tau = 260$ μs). Isolation of the CgTX-sensitive component of the tail current showed that it decayed more slowly, $\tau = 340$ μs, a finding similar to that reported in rat dorsal root ganglion neurons (Regan et al., 1991).

4. Discussion

These results demonstrate a novel action of certain neurosteroids; depression of voltage-gated calcium channels in adult mammalian hippocampal neurons. This effect is rapid and reversible and therefore the target is presumably an interaction either with the cell membrane or the channel protein.

In the absence of CgTX, THCC showed a maximal block at 100 μM; this does not eliminate the possibility that this compound may have a small effect on other components of the Ca^{2+} current. The L-type antagonist nifedipine at concentrations >10 μM shows non-selective effects due to its high lipophilicity (see Regan et al., 1991). A similar effect may be occurring with THCC. However, tail current analysis demonstrated that THCC and CgTX appear to block the same fraction of Ca^{2+} current in these neurons as the effects were non-additive. CgTX has been found to abolish neurotransmitter release in some systems but not others (see Regan et al., 1991) and, as mentioned in the introduction, steroids have also been reported to enhance and depress neurotransmitter release at low and high concentrations respectively. These results therefore suggest that a neurosteroid, such as THCC, could indeed be an endogenous CgTX-like ligand with a role in the regulation of neurotransmitter release.

Certain neurosteroids either potentiate or inhibit the GABA-gated Cl^- current. Of the steroids reported in this study, only DHEAS has been examined with GABA and it was reported to inhibit the GABA-evoked current (Majewska et al., 1990). Neither THCC or pregnanolone have been examined on the GABA-evoked current. Neurosteroid potentiation of GABA, concomitantly with block of voltage-gated Ca^{2+} channels could lead to generalized sedation and/or anesthesia. However, block of GABA and voltage-gated Ca^{2+} channels by DHEAS could depend on the endogenous status and may not necessarily occur concurrently.

Thus, in addition to the potent modulation of the GABA-gated Cl^- channel, the data suggest another site at which the steroids may exert their pharmacological actions to modulate brain excitability. The physiological significance of these findings remains to be elucidated; however, in combination with the modulation of the $GABA_A$ response, depression of voltage-gated calcium channels could contribute to the sedative and anesthetic properties of the steroid metabolites. Studies are ongoing to further characterize the pharmacology and mechanism(s) of action of steroids in the mammalian CNS.

References

Baulieu, E.E. and P. Robel, 1990, Neurosteroids: a new brain function? J. Steroid Biochem. Mol. Biol. 37, 395.

Benoit, E., M.R. Carratu and D. Mitolo-Chieppa, 1988, Interactions between molecules of a steroid anaesthetic (alphaxalone) and

ionic channels of a nodal membrane in voltage-clamped myelinated nerve fibre, Br. J. Pharmacol. 94, 635.

Doerner, D., T.A. Pitler and B.E. Alger, 1988, Protein kinase C activators block specific calcium and potassium current components in isolated hippocampal neurons, J. Neurosci. 8, 4069.

Kay, A.R. and R.K.S. Wong, 1986, Isolation of neurons suitable for patch-clamping from adult mammalian central nervous system, J. Neurosci. Meth. 16, 227.

Majewska, M.D., N.L. Harrison, R.D., Schwartz, J.L. Barker and S.M. Paul, 1986, Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor, Science 232, 1004.

Majewska, M.D., S. Demirgoren, C.E. Spivak and E.D. London, 1990, The neurosteroid dehydroepiandrosterone sulfate is an allosteric antagonist of the $GABA_A$ receptor, Brain Res. 526, 143.

McEwan, B.S., 1991, Non-genomic and genomic effects of steroids on neural activity, Trends Pharmacol. Sci. 12, 141.

Mogul, D.J. and A.P. Fox, 1991, Evidence for multiple types of Ca^{2+} channels in acutely isolated hippocampal CA3 neurons of the guinea-pig, J. Physiol. (London) 433, 259.

Plummer, M.R., D.E. Logthetis and P. Hess, 1989, Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons, Neuron 2, 1453.

Regan, L.J., D.W.Y. Sah and B.P. Bean, 1991, Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and omega-conotoxin, Neuron 6, 269.

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Short communication

Lower efficacy of the dopamine D_1 agonist, SKF 38393, to stimulate adenylyl cyclase activity in primate than in rodent striatum

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The selective D_1 agonist, SKF 38393, stimulated adenylyl cyclase by about 40% of basal activity in rat striatum but by only about 10% in the striatum of rhesus monkeys. In contrast, dopamine stimulated striatal adenylyl cyclase in both species with equal efficiency (70–80%). SKF 38393 30 μM inhibited the effect of 30 μM dopamine by about 45% in rat and by about 75% in primate tissue. This difference may be due to a lower D_1 receptor reserve in primate than in rodent tissue and suggests that only selective D_1 agonists with full efficacy at D_1 receptors can be expected to have beneficial effects in patients with Parkinson's disease.

SK&F 38393; Dopamine D_1 receptors; Adenylyl cyclase; Parkinson's disease; (Rhesus monkey); (Rat)

1. Introduction

The behavioural (motor) effects of striatal dopamine D_1 receptor stimulation differ considerably between rodents and primates. This species difference is revealed inter alia by the dopamine D_1 receptor-selective agonist, SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine). Thus whereas in naive rats D_1 stimulation seems to be a prerequisite for the action of D_2-selective agonists (Braun and Chase, 1986), SKF 38393 has been shown to inhibit the effect of selective D_2 agonists in primates (Bédard and Boucher, 1989). Together with the finding that SKF 38393 was less efficacious than dopamine in stimulating rat striatal adenylyl cyclase (Setler et al., 1978), the above observations suggest that SKF 38393 may be a partial dopamine D_1 receptor agonist, with intrinsic activity being shifted in the primate striatum from D_1 receptor agonistic to D_1 receptor antagonistic activity.

In order to find a possible biochemical correlate for the different behavioural effects of striatal D_1 receptor stimulation in rats and primates, we measured the effect of SKF 38393 on adenylyl cyclase activity in striatum of rats and rhesus monkeys and compared the results with the corresponding effect of dopamine.

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2. Materials and methods

Frozen striatal tissue of male rats of the Chbb:THOM strain *, weighing 320–400 g, and of drug-free (control) rhesus monkeys (two male, two female), aged 12 ± 3 years, weighing 5.9–8.2 kg, was homogenized by hand in a Dounce homogenizer (all glass) in 30-40 volumes of ice-cold 40 mM HEPES-NaOH buffer (pH 7.5 at 22 °C) containing 5 mM $MgCl_2$, 1 mM dithiotreitol and 0.2 mM EGTA. Adenylyl cyclase activity was measured according to the procedure described by Schulz and Mailman (1984) in 100 μl incubation medium containing (mmol/l) 40 HEPES buffer, 5 $MgCl_2$, 1 dithiotreitol, 0.01 EGTA, 0.2 [α-^{32}P]ATP (1 μCi; ICN Biomedicals, Inc.), 0.2 cAMP, 5 phosphocreatine, 0.01 GTP and 25 μg creatine phosphokinase and 0.875 μg adenosine desaminase. The reaction mixture contained 10 μM 1-sulpiride to block D_2 effects of dopamine. The reaction was initiated by adding diluted homogenate with 3–5 μg protein and was carried out at 30 °C for 15 min. The incubation was terminated by adding 100 μl of 3% sodium dodecyl sulfate containing 5 mM ATP. [^{32}P]cAMP was quantitated after separation from [^{32}P]ATP in a high performance liquid chromatographic system equipped with a C18, ODS Ultrasphere 5-μm column (4.6 × 150 mm, Beckman, USA). The mobile phase was 0.15 M

* According to International Index of Laboratory Animals, 3rd edn. 1975, Med. Res. Council, Lab. Animal Center, Great Britain.

sodium acetate, pH 5.0, 17% methanol. Protein content was determined by dye binding using the assay kit provided by Bio-Rad (Richmond, CA, USA).

Statistical calculations were performed using a paired Student's t-test for differences between drug effects in the same species and two-tailed Student's t-test for differences between different species.

3. Results

Dopamine stimulated the activity of adenylyl cyclase in the striatum of rats and the putamen of rhesus monkeys in a concentration-dependent manner with about the same efficiency (80 vs. 70%; open and filled circles in fig. 1) and the same potency ($EC_{50} \pm$ S.E.: 3.1 ± 0.3 vs. 3.0 ± 0.3 μM).

SKF 38393 stimulated the striatal adenylyl cyclase in both species with less efficacy than dopamine, however, this effect was 3-fold greater in rat striatum than in monkey putamen (39 vs. 13%; open and filled triangles in fig. 1). Similarly, the potency of SKF 38393 to stimulate adenylyl cyclase was significantly higher ($P < 0.001$, two-tailed Student's t-test) in rat striatum (EC_{50}: 0.07 ± 0.01 μM) than in the primate putamen (EC_{50}: 0.36 ± 0.04 μM). Compared with that of dopamine, the potency of SKF 38393 was greater in the striatum in both species.

SKF 38393 30 μM, a brain concentration that, assuming equal tissue distribution, would result from a behaviourally active dose of the agonist, blocked the stimulatory effect of the near-maximally active concentration of 30 μM dopamine on adenylyl cyclase activity by about 45% in rat striatum and 75% in monkey putamen (fig. 2).

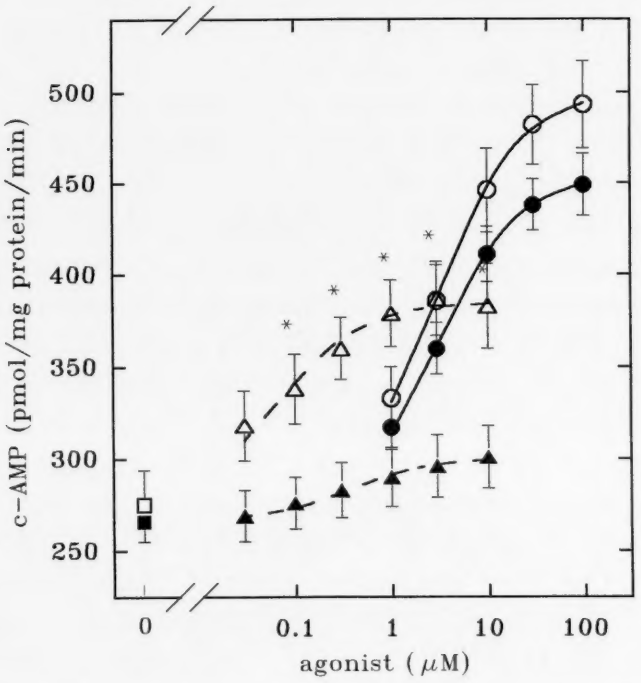

Fig. 1. Adenylyl cyclase activity in striatum of rats (open symbols) and putamen of rhesus monkeys (filled symbols); (□ ■) basal, (○ ●) dopamine-stimulated and (△ ▲) SKF 38393-stimulated activity. The results represent the means ± S.E.M. c-AMP (in pmol/mg protein per min) of four separate experiments, each run in triplicate. * $P < 0.05$ rat vs. monkey by two-tailed Student's t-test.

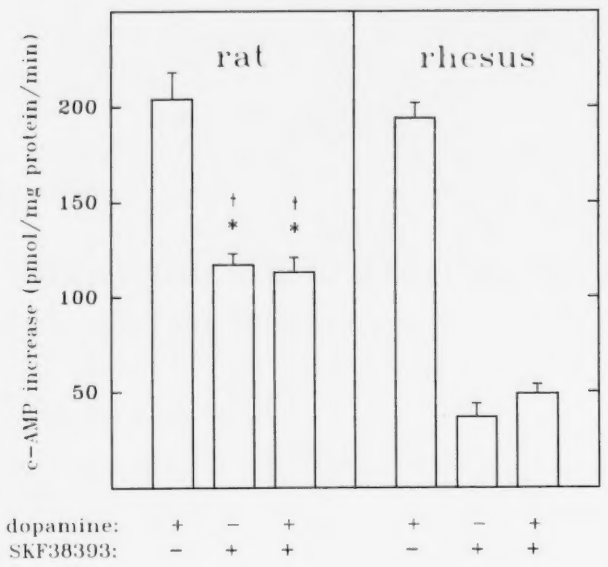

Fig. 2. Partial agonism of SKF 38393 on adenylyl cyclase in striatum of rats (left) and putamen of rhesus monkeys (right). Dopamine 30 μM and SKF 38393 30 μM were used separately and in combination as indicated. The results represent the means ± S.E.M. c-AMP increase (in pmol/mg protein per min) of four separate experiments, each run in triplicate. Basal activities (pmol/mg protein per min ± S.E.M.) were 269 ± 14 in rat striatum and 285 ± 16 in monkey putamen. * $P < 0.001$ rat vs. monkey by two-tailed Student's t-test and † $P < 0.05$ SKF 38393 or dopamine/SKF 38393 vs. dopamine by paired Student's t-test.

The effects of dopamine and SKF 38393 on adenylyl cyclase in monkey caudate nucleus were identical to the effects observed in monkey putamen (data not shown).

4. Discussion

One possible explanation for our observation of marked differences between the rodent and the primate striatum is the assumption that the rat striatum has a greater reserve of D_1 receptors coupled to adenylyl cyclase than does the monkey striatum. In this case the full agonist, dopamine, would be expected to produce the same maximum effect in the striatal tissue in both species whereas, as a partial agonist, SKF 38393 would have a lower efficacy in the tissue with the smaller receptor reserve, i.e. the primate striatum. The radioligand binding data of Madras et al. (1988) indeed show that the primate striatum has a lower density of D_1 receptors than does the rodent striatum (Hess et al., 1986). Consistent with the assumption of spare D_1 receptors in rodent striatum are the results of studies (Battaglia et al., 1986; Arnt et al., 1988) showing that, in rats treated with the irreversible dopamine receptor blocker, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, both the dopamine-stimulated adenylyl cyclase and the D_1 receptor-mediated behaviour are preserved despite blockade of a high percentage of D_1 receptor sites.

Although the difference in receptor reserve between rat and monkey striatum seems sufficient to account for the relative efficacies (maximum effect) of dopamine and SKF 38393 in the two species, this concept appears inadequate to explain the observed differences in potency (EC_{50}) of dopamine and SKF 38393 in rat versus monkey striatum. Theoretically, spare receptors should confer greater potency on a full agonist but hardly affect the potency of a partial agonist (Ariëns et al., 1960). Therefore, the difference in potency between rat striatum (large receptor reserve) and monkey striatum (small receptor reserve) would be expected to be large for dopamine (full agonist) and small for SKF 38393 (partial agonist). Contrary to this prediction, the opposite was true in our experiments. However, this discrepancy can be resolved by taking into account the D_1 receptor affinities of the two agonists in rat vs. monkey striatum. Thus, the higher affinity of the striatal D_1 receptor for dopamine in monkey striatum (Madras et al., 1988) than in rat striatum (Hess et al., 1986) may explain why, according to our observations, the full agonist, dopamine, was about as potent to stimulate the adenylyl cyclase in the monkey despite a lower striatal D_1 receptor reserve, as in rat striatum. Similarly, the higher D_1 receptor affinity of SKF 38393 to rat striatum (Hess et al., 1986) vs. monkey striatum (Madras et al., 1988) may be the reason why this compound was more potent to stimulate striatal adenylyl cyclase in the rat than in the monkey.

Another explanation of the difference between the rat and the monkey striatum we observed would be the assumption of a different ratio of the recently cloned subtypes of the D_1 receptor in the two species (Dearry et al., 1990; Sunahara et al., 1991; Tiberi et al., in press). Since no data are yet available regarding the relative affinities and efficacies of dopamine and SKF 38393 in these D_1 receptor subtypes, it is too early to decide on the validity of this possibility.

Our findings about adenylyl cyclase in rat and monkey striatum bear striking similarities to behavioural observations of these two species from other studies. SKF 38393, while it exerts dopamine agonistic properties in rats by stimulating adenylyl cyclase in vitro (Setler et al., 1978; this study), producing contralateral rotation in the Ungerstedt model and, when combined with a D_2 agonist, inducing stereotypies in naive rats (Braun and Chase, 1986; Setler et al., 1978), behaved like a dopamine antagonist in monkeys by mainly inhibiting dopamine-stimulated adenylyl cyclase in vitro (this study), and the motor effects of a D_2 agonist in naive as well as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine parkinsonian monkeys (Barone et al., 1987; Bédard and Boucher, 1989). Assuming that the behavioural interaction between the striatal D_1 and D_2 receptors as established in rodents also applies to the primate striatum, the inhibitory behavioural effect of the partial agonist, SKF 38393, in primates may be explained by blockade of the endogenous full efficacy dopamine.

In conclusion, the partial D_1 agonist, SKF 38393, had a much lower efficacy to stimulate adenylyl cyclase in primate tissue than in rodent tissue. Differences in striatal D_1 receptor reserve between the rat (high receptor reserve) and the monkey (low receptor reserve) may account for this finding, considering the equal efficacy of the full agonist dopamine in both species. Together with observations from behavioural studies with SKF 38393 in rat versus primate, our present results suggest that only agonists will full intrinsic activity on D_1 receptors may be therapeutically beneficial in human striatal dopamine deficiency conditions such as Parkinson's disease.

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References

Ariëns, E.J., J.M. Van Rossum and P.C. Koopman, 1960, Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested on isolated organs, Arch. Int. Pharmacodyn. 127, 459.

Arnt, J., J. Hyttel and E. Meier, 1988, Inactivation of dopamine D-1 or D-2 receptors differentially inhibits stereotypies induced by dopamine agonists in rats, Eur. J. Pharmacol. 155, 37.

Barone, P., K.S. Bankiewicz, G.U. Corsini, I.J. Kopin and T.N. Chase, 1987, Dopaminergic mechanisms in hemiparkinsonian monkeys, Neurology 37, 1592.

Battaglia, G., A.B. Norman, E.J. Hess and I. Creese, 1986, Functional recovery of D1 dopamine-mediated stimulation of rat striatal adenylate cyclase activity following irreversible receptor modification by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ): evidence for spare receptors, Neurosci. Lett. 69, 290.

Bédard, P.J. and R. Boucher, 1989, Effect of D1 receptor stimulation in normal and MPTP monkeys, Neurosci. Lett. 104, 223.

Brain, A.R. and T.N. Chase, 1986, Obligatory D-1/D-2 receptor interaction in the generation of dopamine agonist related behaviors, Eur. J. Pharmacol. 131, 301.

Dearry, A., J.A. Gingrich, P. Falardeau, R.T. Fremeau, Jr., M.D. Bates and M. Caron, 1990, Molecular cloning and expression of the gene for a human D_1 dopamine receptor, Nature 347, 72.

Hess, E.J., G. Battaglia, A.B. Norman, L.C.Iorio and I. Creese, 1986, Guanine nucleotide regulation of agonist interactions at [^{3}H]SCH 23390-labeled D1 dopamine receptors in rat striatum, Eur. J. Pharmacol. 121, 31.

Madras, B.K., M.A. Fahey, D.R. Canfield and R.D. Spealman, 1988, D1 and D2 dopamine receptors in caudate-putamen of nonhuman primates (*Macaca fascicularis*), J. Neurochem. 51, 934.

Schulz, D.W. and R.B. Mailman, 1984, An improved, automated adenylate cyclase assay utilizing preparative HPLC: Effects of phosphodiesterase inhibitors, J. Neurochem. 42, 764.

Setler, P.E., H.M. Sarau, C.L. Zirkle and H.L. Saunders, 1978, The central effects of a novel dopamine agonist, Eur. J. Pharmacol. 50, 419.

Sunahara, R.K., H.-C. Guan, B.F. O'Dowd, P. Seeman, L.G. Laurier, G. Ng, S.R. George, J. Torchia, H.H.M. Van Tol and H.B. Niznik, 1991, Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1, Nature 350, 614.

Tiberi, M., K.R. Jarvie, C. Silvia, P. Falardeau, J.A. Gingrich, N. Godinot, L. Bertrand, T. Yang-Feng, R.T. Fremeau and M.G. Caron, Cloning, molecular characterization and chromosomal assignment of a gene encoding a novel D_1 dopamine receptor subtype: Differential expression pattern in rat brain compared to the D_{1A} receptor, Proc. Natl. Acad. Sci. U.S.A. (in press).

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Short communication

Loperamide but not morphine has anti-secretory effects in human colon, in vitro

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Isolated preparations of human colonic mucosa were set up in Ussing chambers. Noradrenaline reduced basal short-circuit current, morphine did not nor did loperamide except at the highest concentration tested. Electrically evoked increases in short-circuit current were unaffected by morphine but reduced by loperamide, by a naloxone-insensitive mechanism. Unidirectional fluxes of ^{22}sodium and ^{36}chloride were unaffected by morphine. It is concluded that loperamide but not morphine has an anti-secretory effect on human colonic mucosa.

Morphine; Loperamide; Mucosa; Ion transport; Colon; (Human)

1. Introduction

It seems likely that effects on mucosal electrolyte and water transport contribute to the anti-diarrhoeal actions of opiates in man. Thus loperamide reduced experimentally induced secretion in the human jejunum (Turnberg, 1983) and nufenoxole, a new orally active anti-diarrhoeal agent, demonstrated a naloxone-sensitive inhibition of fluid secretion in the human jejunum (Moriarty et al., 1985). Moreover, morphine inhibited the prostaglandin E_2-induced rise in cyclic AMP levels in homogenates of human colonic mucosa (Rachmilewitz et al., 1983). The purpose of the present investigation was to determine whether morphine and loperamide could affect ion transport mechanisms across isolated preparations of human sigmoid colonic mucosa. A preliminary account of this work has been presented to the British Pharmacological Society (Burleigh, 1990).

2. Materials and methods

2.1. Preparation and electrical stimulation of mucosal tissue

Muscle stripped preparations of human sigmoid mucosa + submucosa were prepared by sharp dissection from specimens of colon resected at operations for removal of colonic tumours. Tissue as taken at a minimum distance of 10 cm from the tumour. Mucosal sheets were mounted in Ussing chambers (0.64 cm^2 window area). Ten millilitres of Krebs bathed each side of the preparation, this was reduced to 5 ml each side for flux experiments. Electrical field stimulation (10 Hz, 5–40 V, 1 ms for 15 s) of mucosal nerves was obtained by passing monophasic pulses across the tissue using a Grass S88 stimulator connected to two aluminium foil electrodes. One foil was placed either side of the preparation to reduce short-circuiting through the bathing fluid (Hubel, personal communication). Stimulus current was calculated from the measured voltage drop across a 10 Ω resistance. It ranged from 1.7 mA (at 5 V) to 31 mA (at 40 V). Some tissue was fixed in 10% phosphate buffered formalin after which haematoxylin and eosin stained sections were prepared for histological examination. Composition of the Krebs fluid was mM: NaCl 118, glucose 11.5, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5.

2.2. Measurement of short-circuit current

Transmucosal short-circuit current was measured with a high impedance voltage clamp (DCV-1000, World Precision Instruments). Compensation was made for fluid resistance between the tips of the voltage electrodes. Both current passing and voltage detecting electrodes utilised a system of Ag/Ag Cl half cells which screw into short large diameter tubes filled with 4% agar in 3 M KCl. Short circuit current was continu-

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ously displayed on pen recorders. All electrical values quoted are calculated for an exposed membrane area of 1 cm^2. Mucosal preparations were allowed 60 min to equilibrate under short-circuit conditions. Drugs were added to the basolateral (serosal) side of the membrane.

2.3. Measurement of radioisotope fluxes

Mucosal to serosal and serosal to mucosal fluxes of $^{22}Na^+$ and $^{36}Cl^-$ were determined on each of the three specimens of colon used for flux experiments. Four mucosal preparations were obtained from each specimen. Unidirectional fluxes of $^{22}Na^+$ and $^{36}Cl^-$ were determined on paired mucosal tissues where individual tissue conductance did not differ by more than 25%. After the equilibration period 2 μCi of $^{22}Na^+$ and 1 μCi of $^{36}Cl^-$ were added to the basolateral side of one tissue and the apical side of its paired tissue. Samples (0.1 ml) of the initially labelled media were taken at the beginning and end of experiments. Samples (0.5 ml) of initially unlabelled media were taken at 10 min intervals for the duration of the experiment. The larger volume samples were immediately replaced with an equal volume of pre-warmed, pre-gassed non-radioactive Krebs fluid. All samples of radioactive media plus some made-up samples containing only $^{22}Na^+$ activity were counted for 10 min in a gamma counter to measure $^{22}Na^+$ activity. Of each sample 0.25 ml was then placed in 5 ml of scintillation fluid and counted for 10 min by liquid scintillation spectrometry, thus total $^{22}Na^+$ and $^{36}Cl^-$ beta radioactivity was determined. The beta counts due to $^{22}Na^+$ were obtained by multiplying the gamma counts by the ratio of $^{22}Na^+$ only beta counts to $^{22}Na^+$ only gamma counts. The sodium beta activity was then subtracted from the total beta activity to give the chloride beta activity. All readings were adjusted for background interference. Ion fluxes were calculated by the method of Schultz and Zalusky (1964). The average short circuit current (in μA) for a 10 min flux collection period was converted to $\mu Eq \cdot cm^{-2} \cdot h^{-1}$ by multiplying by a factor of 0.0583, i.e. $3.6 \times 10^3/AF$ where F is the Faraday constant and A the area of exposed mucosal tissue. Residual ion flux (JR net), which is that part of the short-circuit current (in $\mu Eq \cdot cm^{-2} \cdot h^{-1}$) not accounted for by net movement of Na^+ and Cl^-, was calculated as short-circuit current $- JNa^+$ net $+ JCl^-$ net. Mucosal to serosal fluxes are considered to be positive that is indicative of absorption. Serosal to mucosal fluxes are considered negative that is indicative of secretion.

2.4. Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical differences between means was assessed using the Mann–Whitney U-test for unpaired data and the Wilcoxon matched-pairs signed rank test for paired data. A probability value of less than 0.05 was taken to represent a significant difference.

2.5. Drugs

Drugs used were: loperamide hydrochloride (Janssen, 10 mg dissolved in 0.5 ml propylene glycol to which 9.5 ml distilled H_2O added), morphine sulphate (The Royal London Hospital Pharmacy), naloxone hydrochloride (Sigma) and noradrenaline acid tartrate (Sterling–Winthrop).

3. Results

Mucosal preparations were obtained from 21 specimens of colon resected for distal large bowel tumours and one specimen for carcinoma of caecum. From this specimen two preparations of terminal ileum mucosa and two preparations of ascending colonic mucosa were obtained. Up to four preparations could be set up from each specimen. Twelve of the patients were male and 10 female. The age range was 50–83 years. Histological examination of tissue confirmed the presence of mucosa, muscularis mucosa and submucosa with associated nerve cell bodies. After 60 min equilibration basal (resting) short-circuit current was 117 ± 12 $\mu A \cdot cm^{-2}$ ($n = 75$). Two hours later this value had declined to 78 ± 10 $\mu A \cdot cm^{-2}$.

3.1. Effects of noradrenaline, morphine and loperamide on basal short-circuit current

Cumulative application of noradrenaline, (1.5–360 μM, 3 min contact for each concentration, $n = 7–12$) gave concentration-dependent decreases in short-circuit current which reached a maximum value of 67 ± 9 $\mu A \cdot cm^{-2}$, ED_{50} values was 5.8 μM. Morphine (132 μM) caused a rise in basal short-circuit current (12 ± 4 $\mu A \cdot cm^{-2}$, $n = 6$) which was not prevented by administration of naloxone (2.8 μM, $n = 3$) 15 min before morphine. Loperamide decreased basal short-circuit current at the highest concentration tested (78 μM) giving a fall of 21 ± 5 $\mu A \cdot cm^{-2}$ ($n = 4$).

3.2. Effect of morphine and loperamide on electrically evoked increases in short-circuit current

Electrical field stimulation (10 Hz, 1 ms for 15 s, $n = 20$) of mucosal preparations gave voltage-dependent increases in short-circuit current ranging from 16 ± 3 $\mu A \cdot cm^{-2}$ at 5 V to 49 ± 6 $\mu A \cdot cm^{-2}$ at 40 V. Morphine (26.4 μM, $n = 8$ and 132 μM, $n = 6$, both for 4 min) did not affect the increase in short-circuit

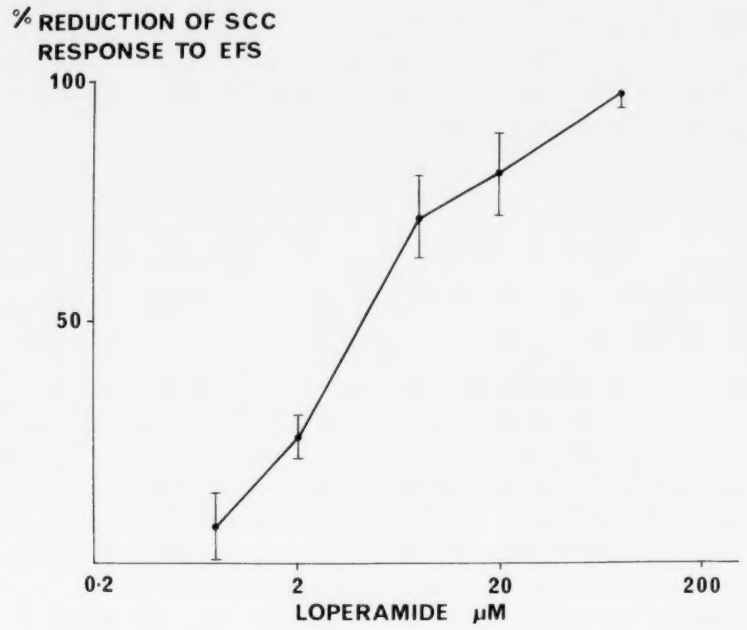

Fig. 1. Effects of loperamide on short-circuit current (SCC) responses of human sigmoid colon mucosal preparations to electrical field stimulation (EFS: 10 Hz, 1 ms, 40 V for 15 s). Each point represents the mean ± S.E.M. from four to six preparations.

current to stimulation. Loperamide (0.8-78 μM, for 30 min) caused a dose-dependent reduction (fig. 1). The effect of loperamide was not due to its solvent, propylene glycol, which did not reduce short-circuit current responses to stimulation (n = 10). Administration of naloxone (2.8 μM) 10 min before loperamide (7.8 μM, n = 6, 19.5 μM, n = 6) did not antagonise its effect. Responses to stimulation of mucosal preparations from the ascending colon (n = 2) and terminal ileum (n = 2) were not reduced on exposure to morphine (132 μM).

3.3. Effects of morphine on ion fluxes

After a 60 min tissue equilibration period $^{22}Na^+$ and $^{36}Cl^-$ were added to the preparations as described in the Methods. A period of 60 min was sufficient to allow ion fluxes to reach a steady state. Morphine (26.4 μM) was then added to the basolateral side of the membrane and the initially unlabelled side sampled twice more at 70 and 80 min. The effect of morphine on ion fluxes was determined by comparing flux measurements during the control period with flux measurements during exposure to morphine. Values of ion fluxes during these periods are given in table 1, it can be seen that morphine had no significant effect.

4. Discussion

As it could not be anticipated whether morphine or loperamide might act on pro-absorptive or anti-secretory mechanisms or both the agents were investigated for their ability to lower basal short-circuit current (pro-absorptive) or reduce electrically evoked increases in short-circuit (anti-secretory). Using both the electrical technique and ion flux measurements the present study shows morphine to have no direct pro-absorptive effect on human colonic epithelial cells. That such effects can be obtained from human mucosal preparations was demonstrated by the concentration-dependent decrease in basal short-circuit current produced by noradrenaline. The positive values for the flux data indicate a net absorption of sodium and chloride ions by the preparation. This finding agrees with the published data of Hubel et al. (1987). The small but significant increase in short-circuit current produced by the higher concentration of morphine cannot be explained, particularly as it was not prevented by prior administration of naloxone.

Electrically evoked increases in short-circuit current were more sensitive to antagonism by loperamide than basal short-circuit current. This may be due to the ability of loperamide to attenuate cholinergic nerve function in the human colon (Burleigh, 1988). Such nerves are responsible for part of the short-circuit current response to electrical stimulation (Hubel et al.,

TABLE 1

Effect of morphine (26.4 μM) on ion transport by human sigmoid colonic mucosa. Fluxes ($\mu Eq \cdot cm^{-2} \cdot h^{-1}$) in the presence of morphine are compared to control fluxes in the absence of the drug.

Control and morphine flux periods timed from addition of $^{22}Na^+$ and $^{36}Cl^-$. ms = mucosa to serosa, sm = serosa to mucosa, unidirectional ion fluxes. JRnet = residual ion flux, i.e. short-circuit current $-JNa^+$ net $+JCl^-$ net. Values expressed as means ± S.E.M., n.s. = non significant, n = 6 pairs of tissue from three specimens of colon.

Group	JNa_{ms}	JNa_{sm}	JNa_{net}	JCl_{ms}	JCl_{sm}	JCl_{net}	JR_{net}	$J_{short\text{-}circuit\ current}$
Control								
50–60 min	5.7 ± 0.5	1.7 ± 0.3	4.0 ± 0.5	12.0 ± 1.3	9.7 ± 0.5	2.3 ± 1.3	0.8 ± 1.6	2.6 ± 0.2
Morphine								
60–70 min	5.9 ± 1.0	2.2 ± 0.2	3.7 ± 0.8	14.0 ± 0.6	10.3 ± 1.5	3.7 ± 0.9	2.8 ± 1.3	2.8 ± 0.2
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Morphine								
70–80 min	5.7 ± 0.4	2.0 ± 0.4	3.7 ± 0.5	13.2 ± 0.7	10.2 ± 1.1	3.0 ± 1.2	2.1 ± 1.3	2.9 ± 0.2
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

1987). The effect on evoked increases in short-circuit current was probably due to a reduction in net secretion of chloride ions, as it is a change in the net movement of chloride ions that is mainly responsible for the increase in short-circuit current produced by electrical stimulation of human colonic mucosa (Hubel et al., 1987). This action was independent of opioid receptor stimulation as it was not reduced by naloxone. The attenuation of cholinergic nerve function in human colon is partly due to non-opioid mechanisms which probably result either from calcium channel blockade, calmodulin inhibition or a combination of both effects (Burleigh, 1988). A positive correlation between inhibition of trifluoperazine binding to calmodulin by loperamide and anti-diarrhoeal activity of the drug been demonstrated by Zavecz et al. (1982). More recently Diener et al. (1988) have shown that the anti-secretory action of loperamide in the rat colon is caused by a block of the calmodulin system. In both these studies and the present investigation, the IC_{50} values for loperamide were similar. Observations made in the present study suggest that loperamide may exert non-opioid, anti-secretory effects in the human colon. Moreover, these effects are exerted locally in the bowel wall and are not secondary to changes in bowel motility.

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References

Burleigh, D.E., 1988, Opioid and non-opioid actions of loperamide on cholinergic nerve function in human isolated colon, Eur. J. Pharmacol. 152, 39.

Burleigh, D.E., 1990, Electrically evoked short circuit current responses in human isolated colonic mucosa are reduced by loperamide but not by morphine, Br. J. Pharmacol. 100, 478P.

Diener, M., S.F. Knobloch and W. Rummel, 1988, Action of loperamide on neuronally mediated and Ca^{2+} or cAMP-mediated secretion in rat colon, Eur. J. Pharmacol. 152, 217.

Hubel, K.A., K. Renquist and S. Shirazi, 1987, Ion transport in human caecum, transverse colon, and sigmoid in vitro. Baseline and response to electrical stimulation of intrinsic nerves, Gastroenterology 92, 501.

Moriary, K.J., D.D.K. Rolston, M.J. Kelly, M. Shield and M.L. Clark, 1985, Nufenoxole, a new anti-diarrhoeal agent, inhibits fluid secretion in the human jejunum, Gut 26, 75.

Rachmilewitz, D., F. Karmeli, M. Chorev and Z. Selinger, 1983, Effect of opiates on human colonic adenylate cyclase activity, Eur. J. Pharmacol. 93, 169.

Schultz, S.G. and R. Zalusky, 1964, Ion transport in isolated rabbit ileum. 1–Short circuit current and Na fluxes, J. Gen. Physiol. 47, 567.

Turnberg, L.A., 1983, Antisecretory activity of opiates in vitro and in vivo in man, Scand. J. Gastroenterol. 18 (Suppl. 84), 79.

Zavecz, J.H., T.E. Jackson, G.L. Limp and T.O. Yellin, 1982, Relationship between anti-diarrhoeal activity and binding to calmodulin, Eur. J. Pharmacol. 78, 255.

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Short communication

α_{1A}-Adrenoceptors in rabbit bronchus

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In the presence of propranolol, bronchial preparations from rabbits were markedly contracted by norepinephrine and phenylephrine but only slightly by clonidine. The contractile responses to norepinephrine were inhibited by prazosin and by high concentrations of yohimbine. The concentration-response curve of norepinephrine was shifted in parallel by WB4101 and 5-methylurapidil but not by 60-min treatment with chloroethylclonidine. These results suggest strongly that norepinephrine-induced contraction of rabbit bronchus is mediated through α_{1A}-adrenoceptors. Furthermore, cyclooxygenase inhibitors did not influence the response to norepinephrine, so norepinephrine-induced contraction is considered not to be mediated through the release of prostaglandins.

Bronchus (rabbit); α_{1A}-Adrenoceptors; WB4101; 5-Methylurapidil

1. Introduction

α_1-Adrenoceptors have no homogenous properties in several tissues (Konno and Takayanagi, 1986). There is increasing evidence that heterogeneity in α_1-adrenoceptors is related to pharmacologically distinct subtypes which may be linked to different second messenger systems (McGrath, 1982). Radioligand binding studies with [^{3}H]prazosin also indicated the existence of two separate populations of α_1-adrenoceptors which were designated α_{1A} and α_{1B}, respectively (Morrow and Creese, 1986). A similar subclassification has been proposed for [^{125}I]BE2254 binding sites on the basis of the affinities of WB4101 and benoxathian and of sensitivity to chloroethylclonidine (Han et al., 1987). The α_{1A}-subtype has a high affinity for WB4101, while the α_{1B}-subtype has a low affinity and is selectively susceptible to chloroethylclonidine.

Little is known about α-adrenoceptors in rabbit bronchus. The results obtained in this study showed that norepinephrine-induced contraction of rabbit bronchus is mediated through α_1-adrenoceptors. Therefore, in the present study we examined which subtype contributes to the norepinephrine-induced contraction of rabbit bronchus.

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2. Materials and methods

Male rabbits (2–3 kg body weight) were killed by a blow on the head and exsanguinated. The main bronchi were dissected free from lung tissue and cut helically. The bronchial preparations (about 30 mm long and 2 mm wide) were suspended in a 20-ml organ bath filled with Krebs solution, kept at 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2, after epithelial cells were removed by 20 gentle rubbings of the luminal surface with a cotton probe. Responses were recorded isotonically under a tension of 0.5 g. The solution contained propranolol (10^{-6} M), ascorbic acid (10^{-3} M), desmethylimipramine (10^{-7} M), corticosterone (3×10^{-5} M) and pargyline (10^{-6} M) to block β-adrenoceptors and to inhibit oxidation and the neural and extraneural uptakes of noradrenaline and monoamine oxidase. The experiments were started after the preparation had been allowed to develop spontaneous tone for 60 min.

The competitive antagonistic activities were expressed as pA_2 values (negative logarithms of the dissociation constant). After determination of control concentration–response curves, the preparations were equilibrated with a competitive antagonist for 20 min. Concentration–response curves were then obtained in the presence of the antagonist and the procedure was repeated with a higher (either 3- or 10-fold) concentration of the antagonist in the same preparation. The pA_2 values were calculated according to the method of Arunlakshana and Schild (1959). In order to test the

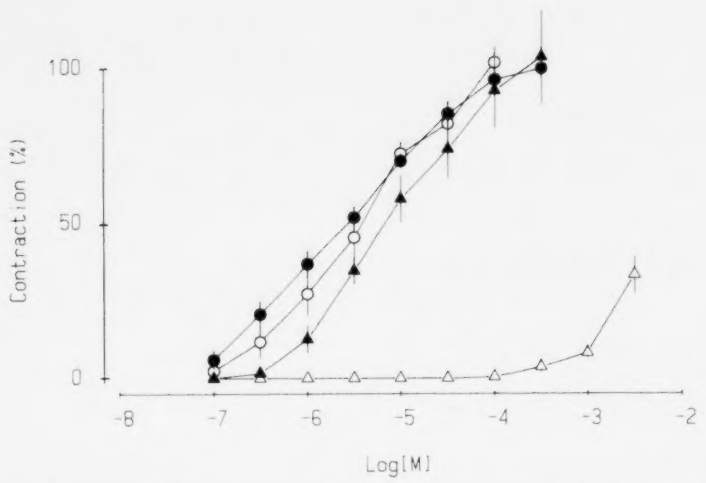

Fig. 1. Concentration–response curves for norepinephrine, phenylephrine and clonidine. Each value is presented as a mean ± S.E. of six experiments. Ordinate: percentage of response to norepinephrine (3×10^{-4} M). Abscissa: logarithm of the molar concentration of agonist. (●) Norepinephrine, (○) norepinephrine after determination of concentration–response curves for all agonists, (▲) phenylephrine and (△) clonidine. Note that the response to norepinephrine (3×10^{-4} M) has no S.E.

effect of chloroethylclonidine on responses to norepinephrine, the preparation was treated with chloroethylclonidine (5×10^{-5} M) for a total of 60 min: following the initial application, the antagonist was renewed every 10 min. The preparation was then allowed to re-equilibrate for 60 min with washing every 10 min.

Calculations and statistics: all numerical data are expressed as means ± S.E. Values were considered to be significant when the P value was less than 0.05 according to Student's t-test or Duncan's new multiple range test as appropriate.

3. Results

Norepinephrine (a non-selective α-adrenoceptor agonist), phenylephrine (an α_1-adrenoceptor agonist) and clonidine (an α_2-adrenoceptor agonist) contracted the bronchial preparations in a concentration-dependent manner (fig. 1). Effective concentrations of clonidine were much higher than those of norepinephrine and phenylephrine. The concentration-response curve of norepinephrine was shifted in parallel by prazosin (10^{-8}, 3×10^{-8} and 10^{-7} M), an α_1-adrenoceptor antagonist and yohimbine (10^{-6}, 3×10^{-6} and 10^{-5} M), an α_2-adrenoceptor antagonist (data not shown). Schild plots of these results yielded straight lines, and a slope near unity (1.05 ± 0.20 for prazosin and 0.95 ± 0.08 for yohimbine), suggesting competitive antagonism. The

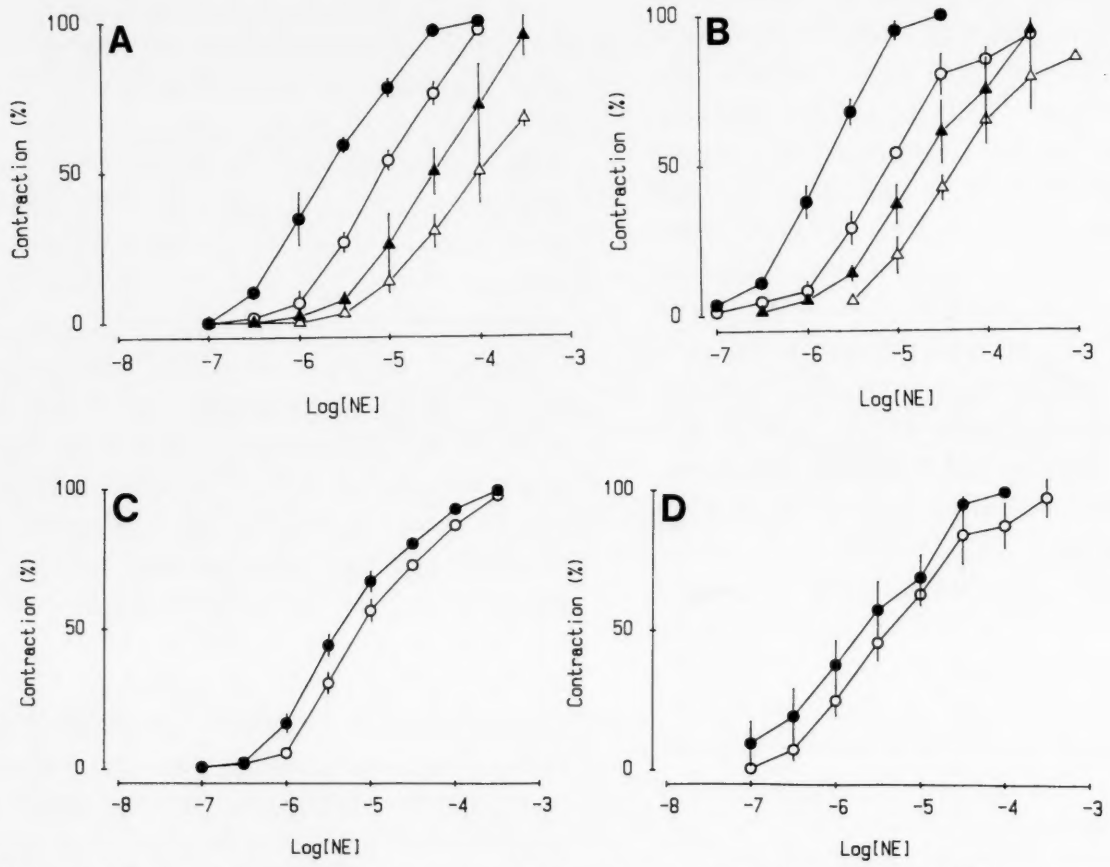

Fig. 2. Effect of WB4101 (A), 5-methylurapidil (B), chloroethylclonidine (C) and indomethacin (D) on concentration–response curve for norepinephrine. Each value is presented as a mean ± S.E. of six experiments. Ordinate: percentage of response to norepinephrine (3×10^{-4} M). Abscissa: logarithm of the molar concentration of norepinephrine. (A) (●) Norepinephrine alone, (○) with WB4101 (3×10^{-9} M), (▲) with WB4101 (10^{-8} M) and (△) with WB4101 (3×10^{-8} M). (B) (●) Norepinephrine alone, (○) with 5-methylurapidil (3×10^{-9} M), (▲) with 5-methylurapidil (10^{-8} M) and (△) with 5-methylurapidil (3×10^{-8} M). (C) (●) Norepinephrine before the treatment with chloroethylclonidine and (○) after the 60-min treatment with chloroethylclonidine (5×10^{-5} M). (D) (●) Before the treatment with indomethacin and (○) after the 60-min treatment with indomethacin (10^{-5} M).

pA_2 values for prazosin and yohimbine were 8.49 ± 0.08 ($N = 12$) and 6.40 ± 0.04 ($N = 9$), respectively.

WB4101 (3×10^{-9}, 10^{-8} and 3×10^{-8} M; fig. 2A), and 5-methylurapidil (3×10^{-9}, 10^{-8} and 3×10^{-8} M; fig. 2B), α_{1A}-selective antagonists, shifted the concentration–response curves for norepinephrine to the right in parallel. Schild plots of the results yielded straight lines with a slope near unity (1.18 ± 0.19 for WB4101 and 1.08 ± 0.29 for 5-methylurapidil; $N = 6$), suggesting competitive antagonism. The pA_2 values for WB4101 and 5-methylurapidil were 9.08 ± 0.17 and 9.15 ± 0.15, respectively. However, 60-min treatment of the bronchial preparation with chloroethylclonidine (5×10^{-5} M), an α_{1B}-inactivating agent, was without any effect on the concentration–response curve for norepinephrine (fig. 2C). The concentration–response curve for norepinephrine was not influenced by 60-min treatment with indomethacin (10^{-5} M; fig. 2D) or phenylbutazone (10^{-4} M).

4. Discussion

Under the experimental conditions we now used contractile responses to adrenoceptor agonists are considered to be mediated only through α-adrenoceptors. Rabbit bronchial preparations were markedly contracted by norepinephrine and phenylephrine but only slightly by clonidine. The pA_2 value of prazosin against norepinephrine obtained was practically equal to that obtained by Takayanagi et al. (1987) for α_1-adrenoceptors in canine portal vein. The pA_2 value of yohimbine against norepinephrine was smaller than those obtained by Takayanagi et al. (1984) for α_2-receptors in electrically stimulated rat vas deferens and guinea-pig ileum, and equal to that reported by Muramatsu et al. (1990) for α_1-adrenoceptors in rabbit thoracic aorta. The present results suggest that rabbit bronchus contains mainly α_1-adrenoceptors.

The 60-min treatment of the preparation with chloroethylclonidine (5×10^{-5} M), which blocks α_{1B}-subtype receptors (Han et al., 1990) did not influence the contraction–response curve for norepinephrine. However, the pA_2 values of WB4101 and 5-methylurapidil were equal to those obtained by Suzuki et al. (1990) for the α_{1A}-subtype receptors in rabbit aorta. These results suggest strongly that norepinephrine-induced contraction of rabbit bronchus is mediated through α_1-adrenoceptors designated α_{1A}. On the other hand, Muramatsu et al. (1990) classified α_1-adrenoceptors into three subtypes (α_{1H}, α_{1L} and α_{1N}). The α_{1H}-subtype is the most sensitive to prazosin (pA_2: > 9.5) and is selectively susceptible to chloroethylclonidine. The α_{1N}-subtype has a higher affinity for HV723 (pA_2: > 9.0) than for prazosin (pA_2: 8.0-9.0), while the α_{1L}-subtype shows low affinity for α_1-adrenoceptor antagonists: the pA_2 values for prazosin, HV723 and WB4101 are 8.0-9.0 and the value for yohimbine was less than 6.4. The results now obtained suggest that the α_{1A}- and α_{1L}-subtypes may have similar pharmacological properties, though further studies are necessary to demonstrate this.

Recently it was reported by Takayanagi et al. (1990, 1991) that norepinephrine interacts with α_2- adrenoceptors and releases prostaglandin $F_{2\alpha}$ to contract guinea pig trachea. We examined the effect of cyclooxygenase inhibitors, indomethacin and phenylbutazone, on the concentration–response curve for phenylephrine to test whether or not norepinephrine-induced contraction of rabbit bronchus is mediated through the release of prostaglandins. As neither of the inhibitors influenced the response to norepinephrine, the possibility was eliminated that prostaglandins are released by norepinephrine.

Acknowledgements

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References

Arunlakshana, O. and H.O. Schild, 1959, Some quantitative uses of drug antagonists, Br. J. Pharmacol. 14, 48.

Han, C., P.W. Able and K.P. Minneman, 1987, Heterogenity of α_1-adrenergic receptors revealed by chloroethylclonidine, Mol. Pharmacol. 32, 505.

Konno, F. and I. Takayanagi, 1986, Characterization of postsynaptic alpha$_1$-adrenoceptors in the rabbit iris dilator smooth muscle, Naunyn-Schmiedeb. Arch. Pharmacol. 333, 271.

McGrath, J.C., 1982, Evidence for more than one type of postjunctional alpha-adrenoceptor, Biochem. Pharmacol. 31, 467.

Morrow, A.L. and I. Creese, 1986, Characterization of α_1-adrenergic receptor subtypes in rat brain: A reevaluation of [^{3}H]WB4101 and [^{3}H]prazosin binding, Mol. Pharmacol. 29, 321.

Muramatsu, I., T. Ohmura, S. Kigoshi, S. Hashimoto and M. Oshita, 1990, Pharmacological subclassification of α_1-adrenoceptors in vascular smooth muscle, Br. J. Pharmacol. 99, 197.

Suzuki, E., G. Tsujimoto, K. Tamura and K. Hashimoto, 1990, Two pharmacological distinct α_1-adrenoceptor subtypes in the contraction of rabbit aorta: Each subtype couples with a different Ca^{2+} signalling mechanism and plays a different physiological role, Mol. Pharmacol. 38, 725.

Takayanagi, I., K. Kawano and K. Koike, 1990, Effect of aging on the response of guinea pig trachea to isoprenaline, Jap. J. Pharmacol. 55, 359.

Takayanagi, I., K. Kawano and K. Koike, 1991, Effects of aging on α_2-adrenoceptor mechanisms in isolated guinea-pig tracheal preparations, Eur. J. Pharmacol. 192, 97.

Takayanagi, I., K. Koike, R. Noguchi, M. Ogishima, F. Takiguchi and T. Sato, 1987, A regional difference in α_1-adrenoceptor mechanism in canine veins, Arch. Int. Pharmacodyn. Ther. 289, 106.

Takayanagi, I., F. Konno, C. Ishii, T. Takemasa, Y. Yanagida, M. Shimizu, H. Mori and H. Sugai, 1984, Action of tizanidine on α_1- and α_2-adrenoceptors in the peripheral tissues, Gen. Pharmacol. 15, 239.

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Short communication

L-N^G-Nitro-arginine inhibits nicotine-induced relaxation of isolated rat duodenum

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We studied the effects of L-N^G-nitro-arginine (L-NOARG), which inhibits nitric oxide (NO) biosynthesis from L-arginine, on the non-adrenergic, non-cholinergic (NANC)-mediated relaxation induced by nicotine in isolated rat duodenum. L-NOARG reduced nicotine-induced relaxation, and L-arginine prevented the inhibitory effect of L-NOARG. However, L-NOARG did not inhibit the tetrodotoxin-insensitive relaxation induced by adenosine 5′-triphosphate, α,β-methyleneadenosine 5′-triphosphate, thyrotropin-releasing hormone or the calcitonin gene-related peptide. Endogenous NO thus could possibly be involved in the NANC-mediated relaxation of rat duodenum induced by nicotine.

Nitric oxide (NO); Nicotine-induced relaxation; Duodenum (rat)

1. Introduction

Nitric oxide (NO), synthesized from L-arginine, has recently been shown to be involved not only in endothelium-dependent vasodilatation but also in relaxation of other types of smooth muscles, including rat and mouse anococcygeous, canine gut and rat gastric fundus (Hobbs and Gibson, 1990; Toda and Okamura, 1990; Bult et al., 1990; Boeckxstaence et al., 1991; Li and Rand, 1990). In a previous study, nicotine was found to induce tetrodotoxin (TTX)-sensitive and non-adrenergic, non-cholinergic (NANC) relaxation in rat duodenum and the relaxation was shown to be partially mediated by peptidergic and purinergic transmission (Irie et al., 1990). Both adenine nucleotides and several neuropeptides, including thyrotropin-releasing hormone (TRH) and calcitonin gene-related peptide (CGRP) were noted to induce TTX-insensitive relaxation of rat isolated duodenum (Tonoue et al., 1979; Furukawa and Nomoto, 1988; Furukawa and Nomoto, 1989). The present study aimed to determine whether endogenous NO is involved in the relaxation of rat duodenum induced by nicotine as well as by adenine nucleotides, TRH and CGRP, using a NO synthase inhibitor, L-N^G-nitro-arginine (L-NOARG) for the purpose.

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2. Materials and methods

Male Wistar-Imamichi rats (9–11 weeks) were used. The animals were killed by stunning. Each duodenum was dissected out and the proximal portion (2.5 cm) was set up in an organ bath containing 5 ml of modified Locke solution (composition in mM; NaCl 154, KCl 4.02, $CaCl_2$, 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 2,97, glucose 5.56, pH 7.0) saturated with 95% O_2 and 5% CO_2 and maintained at 32 °C with continuous bubbling of air. Mechanical activity of the duodenal segments under a load of 1 g was recorded through a Nihonkohden TD-112S isotonic transducer on a Nihonkohden WI-640GS polygraphic recorder. After 1 h equilibration, the segments were exposed to agonists such as nicotine, adenine nucleotides, TRH and CGRP for 2 min at 30-min intervals. The response to each agonist was induced in each preparation. The control response of duodenum to an agonist was observed after 2–3 repeated exposures, and the effect of L-NOARG on the response to the agonist was examined. L-NOARG and L- or D-arginine were added 15 and 20 min, respectively, prior to agonist exposure.

The drugs used were: nicotine tartrate (nicotine, Wako), L-N^G-nitro-arginine (L-NOARG, Ardrich), L-arginine monohydrochloride (L-arginine, Tokyo Kasei), D-arginine monohydrochloride (D-arginine, Wako), adenosine 5′-triphosphate (ATP, Sigma), α,β-methyleneadenosine 5′-triphosphate (α,β-mATP, Sigma), thyrotropin-releasing hormone (TRH) and calcitonin generelated peptide (CGRP, Peptide Institute). All drugs were dissolved in physiological saline.

The data were expressed as means ± S.E.M.. Statistical analysis was done with Student's t-test.

3. Results

Nicotine (1–100 μM) elicited relaxation, which was blocked by both hexamethonium and TTX, of the rat duodenum in a concentration-dependent manner as previously reported (Irie et al., 1990). L-NOARG (10-100 μM) significantly inhibited the nicotine-induced relaxation. L-NOARG (100 μM) inhibited the relaxation elicited by 100 μM nicotine by 56% and that induced by a lower concentration of nicotine, 10 μM, by 87% (table 1). Preincubation with L-arginine (3 or 10 times the concentration of L-NOARG) but not D-arginine partially prevented the inhibitory effect of L-NOARG (fig. 1a, b). Following exposure of the duodenum to L-NOARG, complete recovery of the duodenum response to nicotine failed to occur, even after 30 min incubation in L-NOARG-free medium, and repeated exposure of duodenum to L-NOARG sometimes produced strong inhibition of the nicotine-induced relaxation. Consequently different preparations were used to evaluate the effect of L-arginine on the inhibition by L-NOARG shown in table 1. The inhibitory effect of L-NOARG on the 10 μM nicotine-induced relaxation was dose-related (table 1). The reduction of the nicotine-induced relaxation by L-NOARG was significantly attenuated by L-arginine, but not D-arginine (table 1). Neither L-NOARG nor L- nor D-arginine alone influenced the resting tension of rat duodenum.

We then investigated whether L-NOARG would

TABLE 1

Effects of L-NOARG and L-arginine on relaxant response of isolated rat duodenum to nicotine. Each value was obtained from a different preparation. L-NOARG and L- or D-arginine were added to the medium 15 and 20 min, respectively, prior to nicotine exposure.

Pretreatment	n	Relaxation by nicotine (% of control)		
Nicotine 100 μM				
Saline	5	104 ± 2.2	c	
L-NOARG 100 μM	6	44 ± 4.4		
L-NOARG 100 μM + L-arginine 300 μM	6	68 ± 9.5	a	
Nicotine 10 μM				
Saline	5	99 ± 3.5	c	
L-NOARG 100 μM	10	15 ± 4.8		
L-NOARG 100 μM + L-arginine 1 mM	6	34 ± 6.9	a	NS
L-NOARG 100 μM + D-arginine 1 mM	4	12 ± 5.6		c
L-NOARG 10 μM	5	67 ± 5.1		
L-NOARG 10 μM + L-arginine 100 μM	5	89 ± 3.8		b

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ by Student's t-test. Values are means ± S.E.M.

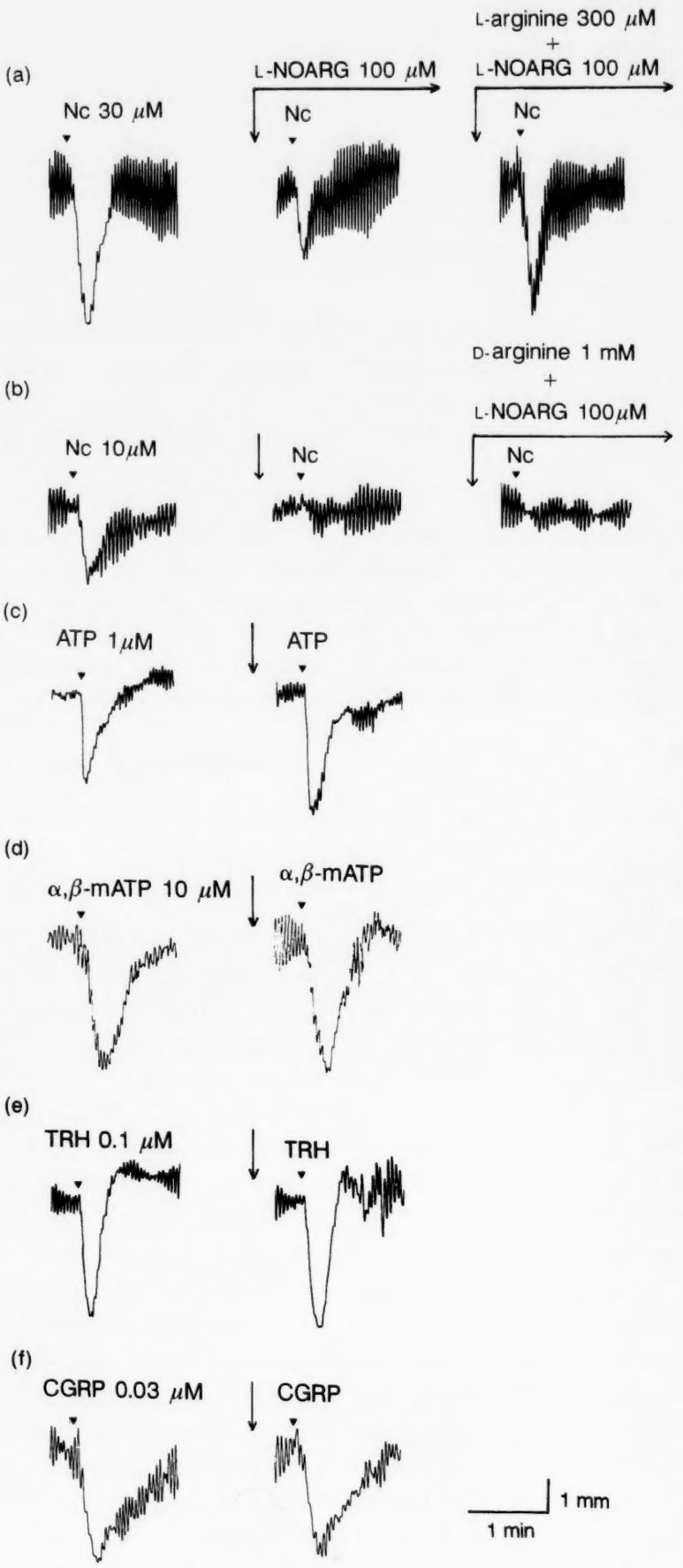

Fig. 1. Effects of L-N^G-nitro-arginine (L-NOARG, 100 μM) and L- or D-arginine (300 μM or 1 mM) on the relaxation of isolated rat duodenum elicited by nicotine (30 μM and 10 μM) (a and b), adenosine 5′-triphosphate (ATP, 1 μM) (c), α,β-methyleneadenosine 5′-triphosphate (α,β-mATP, 10 μM) (d), thyrotropin-releasing hormone (TRH, 0.1 μM) (e) and calcitonin gene-related peptide (CGRP, 0.03 μM) (f). L-NOARG and L- or D-arginine were added to the bath at 15 and 20 min, respectively, prior to agonist exposure.

also inhibit the TTX-insensitive relaxation elicited by ATP, α,β-mATP, TRH or CGRP. All these adenine nucleotides and peptides induced concentration-related and TTX-insensitive relaxation in rat duodenum, as previously reported (Tonoue et al., 1979; Furukawa and Nomoto, 1988; Furukawa and Nomoto, 1989). In contrast to the nicotine-induced relaxation, the relaxation due to ATP (0.3–1 μM), α,β-mATP (10-30 μM), TRH (0.03–0.3 μM) or CGRP (0.03–0.1 μM) (figs. 1c, d, e and f) was not inhibited by L-NOARG (100 μM). The responses to each drug after L-NOARG treatment were: $127 \pm 10.8\%$ (n = 6) of pre-L-NOARG value for 1 μM ATP, $118 \pm 6.3\%$ (n = 4) for 10 μM α,β-mATP, $105 \pm 4.9\%$ (n = 4) for 0.1 μM TRH and $98 \pm 16.0\%$ (n = 6) for 0.03 μM CGRP. The potentiation of the ATP-induced relaxation following L-NOARG treatment was not influenced by L-arginine ($124 \pm 10.3\%$, n = 5).

4. Discussion

L-NOARG inhibited the nicotine-induced relaxation of rat duodenum, and L-arginine, but not D-arginine, attenuated the inhibitory effect of L-NOARG. L-NOARG inhibits NO formation from L-arginine (see Hobbs and Gibson, 1990) and this result thus indicates that endogenous NO is involved in nicotine-induced relaxation of rat doudenum. Data from our previous study indicated that the nicotine-induced relaxation was NANC-mediated and relaxation involved both peptidergic and purinergic transmission since nicotine-induced relaxation was affected by neither guanethidine nor hyoscine, but was partially desensitized by α,β-mATP and inhibited by α-chymotrypsin (Irie et al., 1990). In contrast to the TTX-sensitive relaxation induced by nicotine, the TTX-insensitive relaxation elicited by ATP, α,β-mATP, TRH or CGRP was not inhibited by L-NOARG. Endogenous NO thus appears to be involved in neural transmission. A possible explanation for the differential inhibition by L-NOARG of TTX-sensitive and TTX-insensitive relaxation is that NO may stimulate the release of inhibitory neurotransmitters such as adenine nucleotides and neuropeptides, or NO itself may be a neurotransmitter and co-released with adenine nucleotides or neuropeptides from the duodenum stimulated by nicotine. The endogenous NO system seems to be active only in stimulated neurons, because resting tension was affected by neither L-NOARG nor L-arginine in our study. The reason why the relaxation of duodenum induced by ATP increased after L-NOARG pretreatment is not clear at present.

Gustafsson et al. (1990) reported that the electrically stimulated contraction of guinea-pig ileum, mediated through substance P, was enhanced by L-N^G-monomethyl arginine (L-NMMA), another NO synthase inhibitor, while L-NMMA did not affect the response to exogenous substance P. In this case, endogenous NO was considered to function as an inhibitory modulator of the stimulatory transmitter, substance P.

There are increasingly frequent reports that the NANC-mediated response of other organs is inhibited by NO synthase inhibitors. Li and Rand (1990) report that L-NMMA inhibits NANC relaxation of rat gastric fundus elicited by electrical field stimulation and VIP, with a stronger inhibition of electrically evoked than of VIP-induced relaxation. L-NMMA and L-NOARG also inhibit electrically elicited and NANC-mediated relaxation of mouse and rat anococcygeous (Hobbs and Gibson, 1990), dog duodenum (Toda and Okamura, 1990) and canine gut (Bult et al., 1990; Boeckxstaens et al., 1991). These observations indicate that endogenous NO is quite likely to be involved in neurotransmission. Boeckxstaens et al. (1991) reported that relaxation stimulated electrically, or by ATP or γ-aminobutylic acid is TTX-sensitive and mediated by NANC, is inhibited by L-NMMA. The authors proposed that NO is a neurotransmitter and final mediator of NANC neurons in canine ileum and ileocolonic junctions.

The present results indicate that endogenous NO is quite likely to be involved in NANC-mediated relaxation induced by nicotine in rat duodenum, where NO functions as a neuromodulator or a neurotransmitter. Endogenous NO appears to play an important role in inhibitory neurotransmission among the various types of smooth muscles.

References

Boeckxstaens, G.E., P.A. Pelckmans, H. Bult, J.G. De Man, A.G. Herman and Y.M. Van Maercke, 1991, Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut, Br. J. Pharmacol. 102, 434.

Bult, H., G.E. Boeckxstaens, P.A. Pelckmans, F.H. Jordaens, Y.M. Van Maercke and A.G. Herman, 1990, Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter, Nature 345, 346.

Furukawa, K. and T. Nomoto, 1988, Transition from neurogenic to myogenic response to calcitonin gene-related peptide (CGRP) in the duodenum of developing rat, Jap. J. Pharmacol. 46, 313p.

Furukawa, K. and T. Nomoto, 1989, Postnatal changes in response to adenosine and adenine nucleotides in rat duodenum, Br. J. Pharmaco. 97, 1111.

Gustafsson, L.E., N.P. Wiklund, C.U. Wiklund, B. Cederquist, M.G. Persson and S. Moncada, 1990, Modulation of autonomic neneuroeffector transmission by nitric oxide-like activity in guinea-pig smooth muscle, in: Nitric oxide from L-arginine: a bioregulatory system, eds. S. Moncada and E.A. Higgs (Elsevier, Amsterdam) p. 177.

Hobbs, A.J. and A. Gibson, 1990, L-N^G-Nitro-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus, Br. J. Pharmacol. 100, 749.

Irie, K., T. Nomoto, T. Muraki and K. Furukawa, 1990, Postganglionic neurons in duodenum are non-adrenergic, non-cholinergic in adult rats, while they are cholinergic in neonates, Eur. J. Pharmacol. 183, 2193.

Li, C.G. and M.J. Rand, 1990, Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus, Eur. J. Pharmacol. 191, 303.

Toda, N., H. Baba and T. Okamura, 1990, Role of nitric oxide in non-adrenergic, non-cholinergic nerve-mediated relaxation in dog duodenal longitudinal muscle strips, Jap. J. Pharmacol. 53, 281.

Tonoue, T., K. Furukawa and T. Nomoto, 1979, The direct influence of thyrotropin-releasing hormone (TRH) on the smooth muscle of rat duodenum, Life Sci. 25, 2011.

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Short communication

Dopamine D_2 receptor binding properties of [^{3}H]U-86170, a dopamine receptor agonist

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U-86170F, an imidazoguinolinone, is a potent dopamine D_2 agonist, binding with high affinity to the dopamine D_2 receptor. A K_d of 0.99 nM was determined in membranes from Chinese hampster ovarian (CHO) cells transfected with the D_2 receptor and a K_d of 1.72 nM was obtained in rat striatal homogenates. GTP sensitivity was demonstrated when its addition (300 μM) reduced [^{3}H]U-86170 binding by 60%. This agonist ligand is especially effective in identifying agonists and partial agonists, as well as antagonists, and affords a more precise evaluation of their affinity for the dopamine D_2 receptor, without the use of multiple site analysis, than does an antagonist [^{3}H]-ligand.

Dopamine D_2 receptor agonists; G-proteins; (Cloned receptor)

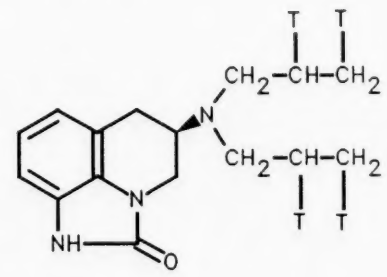
1. Introduction

The dopamine hypothesis has been the major theory regarding schizophrenia. Most attempts to control this disease with pharmacological agents have involved the dopamine system (Carlsson, 1977). Regulation of the activity od dopaminergic neurons by antagonists (antipsychotic drugs) is the major means of controlling the symptoms of schizophrenic patients. Two closely related dopamine D_2 receptors have been cloned (Giros et al., 1989; Chio et al., 1990) and have been shown to belong to the G-protein coupled receptor types. Receptor binding studies using these cloned D_2 receptors have confirmed the findings that the antipsychotic drugs have high affinity for the dopamine receptor. Consequently, receptor binding studies have become a primary means for the characterization and discovery of dopaminergic agents. Several studies have reported on the binding properties of dopamine antagonists, such as spiperone and raclopride, to membranes from cells containing the cloned D_2 receptors. However, to data there have been only a few reports of the use of dopamine agonists as the ^{3}H-ligand in such studies. In this report, the receptor binding properties of [^{3}H]U-86170, a compound which has been shown to have dopamine agonist properties (Lahti et al., submitted), and high affinity for the G-protein coupled high affinity agonist state of the dopamine D_2 receptor, are described.

2. Materials and methods

U-86170 is chemically (R)-(−)-5-(dipropylamino)-5,6-dihydro-4H-imidazo-[5,1-ij]quinolin-2(1H)-one. U-86170F is the monohydrate of the hydrobromide salt of U-86170.

[^{3}H]-86170 (fig. 1) was synthesized by reduction of the N,N-diallyl precursor of U-86170 with carrier-free tritium gas, at ambient temperature and pressure, in



[3H]U–86170

Fig. 1. Structural formula of [^{3}H]U-86170.

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dimethylformamide in the presence of 5% palladium on charcoal catalyst. [^{3}H]U-86170 was purified from the crude reduction mixture by HPLC using a reverse phase C-18 column. Specific activity was determined to be 68 Ci/mmol.

Receptor binding studies with [^{3}H]raclopride (specific activity 80.4 Ci/mmol) were conducted with membranes prepared from Chinese hamster ovarian (CHO) cells stably transfected with the dopamine D_{2i} (Chio et al., 1990), or in homogenates of rat striata (Kohler et al., 1985). Cells were grown to approximately 90% confluence in T150 culture flasks and rinsed twice with TEE (mM: 25 Tris, 5 EDTA, 5 EGTA (pH 7.4)) buffer. The cells were collected in TEE by tapping the flasks, followed by an equal volume rinse of TEE containing 10 mM $MgCl_2$. This was followed by cell disruption using a Polytron (10 s at setting of 6), a 15-min centrifugation at $48\,000 \times g$, resuspension of pellet in TEE with 10 mM $MgCl_2$, via Polytron, a second centrifugation, and resuspension in a buffer containing (mM) 20 HEPES, 10 $MgCl_2$, 150 NaCl, 1 EDTA (pH 7.5). Aliquots of the preparation were stored at $-20\,^\circ$C until used. Protein concentration in the binding assays ranged from 20 to 50 μg/ml. The [^{3}H]raclopride concentration was approximately 1 nM, and non-specific binding was determined using 1 μM haloperidol. For [^{3}H]raclopride binding, which requires a high ion concentration, the incubation buffer contained (mM) 50 Tris, 1 $MgCl_2$, 120 NaCl, 5 KCl, 2 $CaCl_2$ (pH 7.4). Total volume of the incubation mixture was 1.0 ml. After a 1-h, room temperature incubation, samples were filtered over Schleicher & Schuell No. 24 filters (pretreated with 0.05% PEI), and rinsed three times with approximately 3 ml of 50 mM Tris (pH 7.4) buffer/rinse. Filters were counted using standard liquid scintillation techniques.

Saturation studies with [^{3}H]U-86170 were conducted using both D_2 receptor sources (cloned receptor and striatal tissue), and radioligand concentrations ranged from 0.9 to 13.8 nM. U-86170F, 1 μM, was used as the non-specific binding determinant. Competitive binding studies were conducted using a ligand concentration of approximately 2 nM [^{3}H]U-86170 with 1 μM U-86170F for a non-specific binding determinant and for [^{3}H]raclopride a concentration of approximately 1 nM was used with 1 μM haloperidol serving as the non-specific binding determinant. IC_{50} values were converted to K_i values using standard methods (Cheng and Prusoff, 1983).

3. Results

Saturation studies demonstrated that in the CHO–D_{2i} cell preparations, [^{3}H]U-86170 had a K_d of 0.99 ± 0.02 nM and in rat striatal homogenates, the K_d was 1.72 ± 0.07 nM. This higher value in rat striata may be a reflection of receptor heterogeneity in this tissue. The B_{max} values in the CHO–D_{2i} membranes was 1.95 ± 0.15 pmol/mg protein, and in rat striata it was 0.19 ± 0.03 pmol/mg protein. [^{3}H]Raclopride was reported (Lahti et al., submitted) to have a K_d of 0.64 nM and a B_{max} of 2.8 pmol/mg protein in membranes obtained from CHO–D_{2i} cells. The agonist character of [^{3}H]U-86170 binding to CHO–D_{2i} membranes was demonstrated by the finding that the addition of 300 μM GTP caused a 60% reduction in binding.

TABLE 1

Inhibitory effect (K_i (nM) ± S.D.) of various compounds on dopamine D_2 agonist ([^{3}H]U-86170) and antagonist ([^{3}H]raclopride) binding in different receptor preparations. [^{3}H]U-86170 was used at approximately 2 nM and 1 μM U-86170F was used for non-specific binding. [^{3}H]Raclopride was used at 1 nM and 1 μM haloperidol was used to determine non-specific binding. Drugs were tested at four to six different concentrations in triplicate. Incubation was for 1 h at room temperature; samples were filtered, rinsed, and counted. IC_{50} values were converted to K_i values using standard methods.

Compound	CHO–D_{2i} [^{3}H]U-86170	Rat striata	
		[^{3}H]U-86170	[^{3}H]Raclopride
Dopamine	4.6 ± 1.8	6.7 ± 2.2	336.0 ± 132
U-68553B	0.96 ± 0.34	2.7 ± 0.95	6.4 ± 0.85
HW-165	6.4 ± 1.2	14.2 ± 3.6	37.7 ± 6.6
(−)-3-PPP	46.3 ± 11.2	79.8 ± 17.8	130.0 ± 38.2
Clozapine	63.0 ± 12.5	262.3 ± 27.4	152.0 ± 68.5
Haloperidol	0.27 ± 0.06	2.4 ± 0.6	1.8 ± 0.41
AJ-76	202.6 ± 61.0	665.5 ± 127.2	144.1 ± 30.7
UH-232	18.3 ± 5.5	19.8 ± 11.8	10.4 ± 2.2
SDZ 208-912	1.7 ± 0.43	8.1 ± 4.0	0.47 ± 0.08

A comparison of the inhibitory effect of various compounds on [^{3}H]U-86170 binding in CHO–D_{2i} cells and rat striata, and with [^{3}H]raclopride in rat striata brings out some interesting findings (table 1). [^{3}H]U-86170 binding in the CHO–D_{2i} cell preparation produces the lowest K_i values for most compounds. The three exceptions are the antagonists AJ-76 and UH-232, and the low intrinsic activity SDZ 208–912. The results also demonstrate the value in using the cloned CHO–D_{2i} receptor membrane preparation for more definitive binding results for agonist and partial agonists.

A comparison of the inhibitory effect of the compounds on [^{3}H]U-86170 and [^{3}H]raclopride in rat striata is also informative (table 1). The full agonists, such as dopamine and U-68553B (Lahti et al., 1989), are much more active against the agonist ligand, [^{3}H]U86170, than against the antagonist ligand, [^{3}H]raclopride. Partial agonists, such as HW-165 (Hjorth et al., 1986) and (−)-3-(3-hydroxyphenyl)-N-propylpiperidine ((−)-3-PPP) (Hjorth et al., 1983) also have a slightly lower affinity for the agonist ligand. Low intrinsic activity partial agonists like SDZ-208-912 (Coward et al., 1990) exhibit an inhibition profile comparable to antagonists such as haloperidol, clozapine, AJ-76

and UH-232 (Svensson et al., 1986), by having a lower K_i versus the antagonist ligand.

4. Discussion

The results presented in this report demonstrate the utility of [^{3}H]U-86170 as a ligand in receptor binding studies at the D_2 receptor. The results also demonstrate the greater overall sensitivity and accuracy in using an agonist ligand, compared to an antagonist ligand, for the determination of binding affinities of agonists and partial agonists. Although the use of an antagonist ^{3}H-ligand and multiple site analysis for resolution into high- and low-affinity components will yield accurate affinities for full agonists. The use of cloned receptors in binding studies adds another dimension of accuracy in drug characterization by eliminating the possibility of multiple binding to different receptors or to receptor subtypes. We conclude that [^{3}H]U-86170 is a dopamine agonist with high affinity for the dopamine D_2 receptor and its use in receptor binding assays with cloned dopamine D_2 receptor preparations represents an improvement in the evaluation of dopaminergic agents.

Acknowledgements

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References

Carlsson, A., 1977, Antipsychotic drugs, neurotransmitters, and schizophrenia, Am. J. Psychiat. 135, 164.

Cheng, Y.-C. and W.H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Chio, L.C., G.F. Hess, R.S. Graham and R.M. Huff, 1990, A second molecular form of D_2 dopamine receptor in rat and bovine caudate nucleus, Nature 343, 266.

Coward, D.M., A.K. Dixon, S. Urwyler, T.G. White, A. Ena, M. Karobath and G. Shearman, 1990, Partial dopamine-agonistic and atypical neuroleptic properties of the amino-ergolines SDZ 208-911 and SDZ 208-912, J. Pharmacol. Exp. Ther. 252, 279.

Giros, B., P. Sokoloff, M.-P. Martes, J.-F. Riou, L.J. Emorine and J.C. Schwartz, 1989, Alternative splicing directs the expression of two D_2 dopamine receptor isoforms, Nature 342, 923.

Hjorth, S., A. Carlsson, D. Clark, K. Svensson, H. Wikstrom, D. Sanchez, P. Lindberg, U. Hacksell, L.-E. Arvidsson, A. Johansson and J.L.G. Nilsson, 1983, Central dopamine agonist and antagonist actions of the enantiomers of 3-PPP, Psychopharmacology 81, 89.

Hjorth, S., K. Svensson, A. Carlsson, H. Wikstrom and B. Andersson, 1986, Central dopaminergic properties of HW-165 and its enantiomers; Trans-octahydrobenzo(f)-quinoline congeners of 3-PPP, Naunyn-Schmiedeb. Arch. Pharmacol. 333, 205.

Kohler, C., H. Hall, S.-V. Ögren, G. Gawell, 1985, Specific in vitro and in vivo binding of ^{3}H-raclopride, Biochem. Pharmacol. 34, 2251.

Lahti, R.A., M.F. Piercey, D.L. Evans, K.J. Carrigan, G.L. Neff, C.B. Barsuhn and G. Vogelsang, 1989, U-68553B. A novel dopamine autoreceptor agonist with chronic activity, Soc. Neurosci. Abstr. 15, 270 (Abstract No. 109.18).

Svensson, K., A.M. Johansson, T. Magnusson and A. Carlsson, 1986, (+)-AJ-76 and (+)UH-232: Central stimulants acting as preferential dopamine autoreceptor antagonists, Naunyn-Schmiedeb. Arch. Pharmacol. 334, 234.

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Rapid communication

High-affinity binding of [^{3}H]1-methyl-4-phenyl-2,3-dihydropyridinium ion to mouse striatal membranes: putative vesicular location

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Systemic exposure to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) produces a Parkinson-like disorder in man and in primates (Langston et al., 1983). The toxicity of MPTP may result from its biotransformation to MPP^+ (1-methyl-4-phenyl-2,3-dihydropyridinium ion) by type B MAO. MPP^+ is actively transported into dopaminergic neurons via the catecholamine transporter (Javitch et al., 1985). Whereas neuronal uptake of MPP^+ appears to be one of the steps required for its neurotoxicity, the site of its long-term storage in the brain of sensitive species is unknown.

Though neuromelanin in primate brain (D'Amato et al., 1986) and MAO-A in mouse brain (Del Zompo et al., 1990) has recently been shown to bind MPP^+, the presence of an intracellular depot for the toxic metabolite in dopaminergic neurons cannot be excluded. The present study provides the first evidence that [^{3}H]MPP^+ associates with high-affinity at a striatal site not MAO- or neuromelanin-linked.

Fresh striatal tissue, homogenized (1:40 w/v) in cold 50 mM Tris HCl buffer, (pH 7.30) containing 5 mM KCl and 120 mM NaCl, using a smooth glass-glass homogenizer, was centrifuged at 48 000 × *g* for 10 min at 4 °C. After incubation at 37 °C for 10 min, the resuspended pellets were centrifuged twice at 48 000 × *g* for 10 min at 4 °C. The final pellets were suspended (1:40 w/v) in cold buffer supplemented with 10 μM pargyline to prevent any binding of [^{3}H]MPP^+ to MAO-A (Del Zompo et al., 1990) and 50 μM ascorbate. Triplicate tissue aliquots (approx. 150 μg protein) were incubated for 10 min at 37 °C with 1 nM or 0.12–5 nM [^{3}H]MPP^+ (78.2 Ci/mmol, Dupont-NEN) in the absence or presence of competing drugs, and of 10 μM dopamine to characterize non-specific binding. The samples were then filtered through Whatman GF/B filters presoaked in 0.5% polyethylenimine and washed. Saturation and competition parameters were calculated using the LIGAND program.

Whereas no specific binding of [^{3}H]MPP^+ could be detected at 4 °C the incubation of striatal membranes at 37 °C allowed the measurement of a specific, saturable and high-affinity binding of the neurotoxin, with mean K_D and B_{max} values of 1.4 ± 0.4 nM and 168 ± 15 fmol/mg protein (n = 4), respectively.

The striatum, among several brain areas, contained the highest density of [^{3}H]MPP^+ binding sites; no binding was found in the cerebellum or in membrane fractions from peripheral tissues such as lung, liver, stomach, kidney, heart and the adrenal gland. The unilateral, intrastriatal injection of 6-hydroxydopamine (6-OHDA, 16 μg/4 μl of saline containing 0.05% ascorbate, A 1, L 2.2, V 3.2) reduced the maximum number of [^{3}H]MPP^+ sites by 70 ± 5% (n = 3), compared to the intact, contralateral striatum, while leaving unchanged the binding affinities. The results showed that the MPP^+ neurotoxin can bind with high affinity to a population of sites mainly localized in dopaminergic nerve endings of the mouse striatum, the brain area most sensitive to MPP^+ toxicity.

It is noteworthy that dopamine, tyramine and amphetamine potently displaced specifically bound [^{3}H]MPP^+ in the striatum (table 1), and that reserpine and tetrabenazine, two well-recognized ligands for the vesicular monoamine transporter, were fairly potent inhibitors of [^{3}H]MPP^+. Mazindol, debrisoquine and chloroquine were poor inhibitors of this binding, indicating that, under the assay conditions we used, neither the neuronal dopamine transporter, nor neuromelanin, nor MAO enzymes are preferential targets for MPP^+. Furthermore, tyramine binds at a striatal vesicular site where it is involved in the transport process of dopamine (Vaccari, 1986).

In conclusion, the present results are consistent with

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TABLE 1

Inhibition of [^{3}H]MPP$^+$ binding. [^{3}H]MPP$^+$ (1 nM) was incubated 10 min with mouse striatal membranes and with 7 concentrations of each compound in triplicate. Non-specific binding, 8% of the total, was the same for all drugs tested. K_i values were determined with the LIGAND program, based on a K_D value of 1.12 nM for [^{3}H]MPP$^+$. The values are means ± S.E. from two assays. Other drugs that did not inhibit specific [^{3}H]MPP$^+$ binding to mouse striatum homogenate include (−)-butaclamol, (+)-butaclamol, apomorphine, L-sulpiride, guanethidine, teophilline, caffeine, verapamil, MK 801 and adenosine.

Compound	K_i (nM)
Dopamine	5.6 ± 0.6
Tyramine	6.5 ± 1
Amphetamine	22 ± 4
Norepinephrine	107 ± 7
Reserpine	121 ± 5
Tetrabenazine	143 ± 9
MPTP	826 ± 72
Debrisoquine	1125 ± 120
Mazindol	1177 ± 140
Chloroquine	> 10000

a vesicular location of high-affinity [^{3}H]MPP$^+$ binding sites in dopaminergic nigrostriatal terminals of the mouse, a MPTP-sensitive species.

Thus, MPP$^+$, under appropriate assay conditions, may represent a marker of striatal dopamine storage vesicles.

References

D'Amato, R.J., Z.P. Lipman and S.H. Snyder, 1986, Selectivity of parkinsonian neurotoxin MPTP: toxic metabolite MPP$^+$ binds to neuromelanin, Science 231, 987.

Del Zompo, M., S. Ruiu, R. Maggio, M.P. Piccardi and G.U. Corsini, 1990, [^{3}H]1-methyl-4-phenyl-2,3-dihydropyridinium ion binding sites in mouse brain: pharmacological and biological characterization, J. Neurochem. 54, 1905.

Javitch, J.A., R.J. D'Amato, S.M. Strittmatter and S.H. Snyder, 1985, Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity, Proc. Natl. Acad. Sci. U.S.A. 82, 2173.

Langston, J.W., P. Ballard, J.W. Tetrud and I. Irwin, 1983, Chronic parkinsonism in humans due to a product of meperidine-analog synthesis, Science 219, 979.

Vaccari, A., 1986, High affinity binding of ^{3}H-tyramine in the central nervous system, Br. J. Pharmacol. 89, 15.

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Announcement

Recent Advances in Receptors Characterisation, Classification and Nomenclature – Therapeutic Implications

London (U.K.), October 28–29, 1991

Further information from: IBC Technical Services, Gilmoora House, 57-61 Mortimer Street, London W1N 7TD, U.K. Tel. 44.71.637 4383, fax 44.71.631 3214.

EJP 20925

Announcement

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Pisa (Italy), May 14–16, 1992

Scientific Secretariat: Dr. C.A. Maggi, Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131 Florence, Italy.
Organizing Secretariat: Fondazione Internazionale Menarini, Piazza del Carmine 4, 20121 Milan, Italy.

EJP 20926

Announcement

The Role of Serotonin in Psychiatric Disorders

Castres (France), June 24–26, 1992

This international symposium will be organised by Mike Briley and David Grahame-Smith.

Further details may be obtained from: Mrs Martine Dehaye, Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, 81100 Castres, France. Tel. 33.63.71.42.64, telex 531635, fax 33.63.35.66.29.

EJP 20927

Announcement

7th International Catecholamine Symposium

Amsterdam (The Netherlands), June 22–27, 1992

The 7th International Catecholamine Symposium will be held in Amsterdam, The Netherlands, 22–27 June 1992. The symposium will cover all aspects of catecholamine research, from basic to clinical.

If you missed the first announcement and want to receive a copy of the Provisional Program and Call for Papers *please write to*:
7th International Catecholamine Symposium, c/o Conference Bureau Free University, De Boelelaan 1105, 1081 HV Amsterdam, The Netherlands. Tel. 31.20.548 4656, fax 31.20.646 2425.

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The EUROPEAN JOURNAL OF PHARMACOLOGY publishes manuscripts on the mechanisms of action of chemical substances affecting biological systems.
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Acceptability of rapid communications will be decided by the editors of the journal, who will be advised by an external referee. Full length manuscripts and short communications are generally submitted to 2 referees who are chosen for their ability to evaluate the work. Authors may request that certain referees should not be chosen. Members of the Editorial Board will usually be called upon for advice when there is disagreement among the referees or between referees and authors, or when the editors believe that the manuscript has not received adequate consideration from the referees. In order to reduce mailing cost, manuscripts will not be returned. Only the decision of the editorial office, together with the advice of the referees will be sent to the author(s). Referees will be requested to keep the copy of the manuscript which they have received for evaluation, and to destroy it once they have been notified of the final decision concerning the manuscript. When authors submit a revised manuscript, they should reply to the comments of the referees, indicating which comments have been incorporated and the reasons for disregarding any suggestions considered unacceptable. Revised manuscripts should be returned in duplicate to the editors. A revised manuscript should be received by the editorial office not later than 5 weeks (in case of minor revision) or 10 weeks (in case of extensive revision) after the editorial decision was sent to the author(s); otherwise it will be processed as a new manuscript. Handwritten additions or corrections will not be accepted. Only complete retyping of the pages affected by revision is acceptable.

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For extensive 'Notice to authors' see Vol. 192, No. 1, p. vii.
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P.O. Box 5023, 3502 JA Utrecht, The Netherlands,
or: Vondellaan 6, 3521 GD Utrecht, The Netherlands

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(e) Rapid communications have priority at the editorial office and publishers.
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Sample references

periodicals:

Carlson, J.H., D.A. Bergstrom and J.R. Walters, 1987, Stimulation of both D_1 and D_2 dopamine receptors appears necessary for full expression of postsynaptic effects of dopamine agonists: a neurophysiological study, Brain Res. 400, 205.

books:

Howard, C.R., 1986, Arena Viruses (Elsevier, Amsterdam) p. 89.

De Wied, D., 1987, Neuropeptide generation, CNS effects and mode of action, in: Pharmacology, Int. Congr. Series, Vol. 750, eds. M.J. Rand and C. Raper (Excerpta Medica, Amsterdam) p. 173.

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